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The molecular epidemiology of *Mycobacterium tuberculosis* and *Mycobacterium avium* complex infections in HIV seropositive patients from South East England.

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THE MOLECULAR EPIDEMIOLOGY OF *MYCOBACTERIUM TUBERCULOSIS*
AND *MYCOBACTERIUM AVIUM* COMPLEX INFECTIONS IN
HIV SEROPOSITIVE PATIENTS FROM SOUTH EAST ENGLAND

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ABSTRACT

Opportunistic infections with *Mycobacterium tuberculosis* and *M. avium* complex (MAC) are common among HIV patients. This study used molecular typing techniques on isolates from such patients, referred by laboratories in South East England between 1991 and 1994, to investigate the epidemiology of these infections within the region.

IS6110 restriction fragment length polymorphism analysis was satisfactory in terms of typability, discriminatory power and reproducibility, for typing *M. tuberculosis* isolates from 96 HIV patients and 84 controls. The viability of strains stored at room temperature for more than two years was low, however. Isolates from HIV patients were more often from extrapulmonary sites, compared to those from controls, and resistance to antituberculous drugs occurred more frequently in the former.

Considerable heterogeneity was observed among the isolates from HIV patients, reflecting the diverse origins of such patients living in the region. However, four clusters of cases with indistinguishable strains were also detected, significantly more than among the controls. This suggests that recently transmitted infection is responsible for clinical disease more frequently among HIV patients, consistent with findings elsewhere in the world. In one cluster, epidemiological information supported the conclusion that nosocomial transmission of tuberculosis had occurred from one patient to two others, and possibly to a healthcare worker. This was the first outbreak of tuberculosis in an HIV care centre described in the United Kingdom.

Pulsed-field gel electrophoresis of *Ase* I large restriction fragments was a highly discriminatory method for typing MAC isolates from 56 AIDS patients with disseminated infection. Strains from different patients were markedly heterogeneous. The majority of patients with infection at more than one body site, as well as those with infection persisting for twelve months or more, had the same strain throughout. A cluster of indistinguishable strains was observed, although insufficient epidemiological information was available to conclude that this was an outbreak.

CONTENTS

	Page
ABSTRACT	2
Acknowledgements	7
Abbreviations	8
List of Tables	9
List of Figures and Plates	10
1 INTRODUCTION	11
1.1 Mycobacterial infections in HIV patients	12
1.2 <i>Mycobacterium tuberculosis</i>	14
1.2.1 Trends in tuberculosis notifications and the contribution of HIV co-infection	14
<i>Developing countries</i>	14
<i>United States of America</i>	15
<i>Western Europe</i>	16
<i>United Kingdom</i>	16
1.2.2 Tuberculosis in patients with HIV infection	19
1.2.3 Typing methods for <i>M. tuberculosis</i>	24
<i>Restriction fragment length polymorphism analysis</i>	25
<i>IS6110 and its role in studies of molecular epidemiology</i>	26
<i>Other repetitive DNA elements</i>	36

<i>Application of the polymerase chain reaction to typing</i>	39
1.3 <i>Mycobacterium avium</i> complex	44
1.3.1 Infections caused by <i>M. avium</i> complex	44
1.3.2 Microbiology of the <i>M. avium</i> complex	45
1.3.3 Environmental sources and pathogenesis	46
1.3.4 Virulence factors	49
1.3.5 Clinical features of disseminated <i>M. avium</i> complex infection	50
1.3.6 Typing methods for <i>M. avium</i> complex	51
<i>Methods based on phenotype</i>	51
<i>Restriction fragment length polymorphism analysis</i>	52
<i>Separation of large restriction fragments by pulsed field gel electrophoresis</i>	54
1.4 Scope of the study	57
 2 METHODS	 61
 2.1 <i>Mycobacterium tuberculosis</i>	 62
2.1.1 Identification of <i>M. tuberculosis</i>	62
2.1.2 Susceptibility testing	64
2.1.3 Subculture of stored strains	65
2.1.4 Restriction fragment length polymorphism analysis	65
<i>Preparation of chromosomal DNA</i>	66
<i>Restriction digestion of chromosomal DNA</i>	66

	<i>Electrophoresis and Southern transfer of DNA fragments</i>	67
	<i>Preparation of IS6110 probe by PCR</i>	68
	<i>Hybridisation</i>	69
	<i>Chemiluminescent detection of IS6110 probe</i>	70
	<i>Preparation and detection of internal size markers</i>	70
2.1.5	Computer analysis of IS6110 restriction fragment length polymorphism patterns	71
2.2	<i>Mycobacterium avium complex</i>	73
2.2.1	Selection, identification and storage of isolates	73
2.2.2	Separation of large restriction fragments by pulsed-field gel electrophoresis	73
	<i>Subculture of isolates</i>	73
	<i>Preparation of agarose plugs and liberation of intact mycobacterial DNA</i>	74
	<i>Restriction endonuclease digestion</i>	74
	<i>Pulsed-field gel electrophoresis</i>	75
3	RESULTS	76
3.1	<i>Mycobacterium tuberculosis</i>	77
	Viability of stored strains	77
	Numbers of cases and controls	78
	Sites of infection	81
	Drug susceptibility	82

Restriction fragment length polymorphism analysis	83
<i>HIV seropositive patients</i>	83
<i>Characteristics of a cluster of patients with indistinguishable strains</i>	89
<i>Control patients</i>	95
3.2 <i>Mycobacterium avium</i> complex	97
4 DISCUSSION	106
4.1 <i>Mycobacterium tuberculosis</i>	107
4.2 <i>Mycobacterium avium</i> complex	121
5 CONCLUSIONS	131
REFERENCES	136

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ABBREVIATIONS

AIDS	Acquired Immunodeficiency Syndrome
BCG	Bacille Calmette-Guerin
CDC	Centers for Disease Control
CDSC	Communicable Disease Surveillance Centre
CHEF-DR	Clamped Homogeneous Electric Fields with Dynamic Regulation
CMV	Cytomegalovirus
DNA	Deoxyribonucleic acid
DR	Direct Repeat
EDTA	Ethylene diamine tetra acetic acid
HIV	Human Immunodeficiency Virus
LRF	Large Restriction Fragments
MAC	<i>Mycobacterium avium</i> complex
MIC	Minimum Inhibitory Concentration
MDRTB	Multidrug resistant tuberculosis
MPTR	Major Polymorphic Tandem Repeat
OADC	Oleic acid, albumin, dextrose, catalase
PCP	<i>Pneumocystis carinii</i> pneumonia
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
PGRS	Polymorphic GC-rich repetitive sequence
PHLS	Public Health Laboratory Service
PNBA	p-nitro benzoic acid
RAPD	Random amplified polymorphic DNA analysis
RFLP	Restriction fragment length polymorphism
SSC	Sodium chloride, sodium citrate solution
TBE	Tris Borate EDTA
TBS	Tris-buffered saline
TCH	Thiophen-2 carboxylic acid hydrazide
TE	Tris EDTA solution
WHO	World Health Organisation

List of Tables

	Page
1.2.1 Summary of published community studies of tuberculosis epidemiology using IS6110 RFLP analysis (1991-95).	30
1.2.2 Repetitive DNA elements in <i>M. tuberculosis</i> .	36
2.1.1 Identification of <i>M. tuberculosis</i> subspecies.	63
3.1.1. Viability of stored isolates of <i>M. tuberculosis</i> referred from HIV seropositive patients.	77
3.1.2 Laboratories from which <i>M. tuberculosis</i> isolates used in this study were referred.	79
3.1.3 Presumed ethnic origins of HIV positive cases and controls.	80
3.1.4 Clinical sites from which <i>M. tuberculosis</i> was isolated in HIV positive patients and controls.	81
3.1.5 Susceptibility of <i>M. tuberculosis</i> isolated from HIV patients and controls to first line antimycobacterial drugs.	82
3.1.6 Clusters of cases with indistinguishable strains of <i>M. tuberculosis</i> on RFLP analysis.	87
3.2.1 Clinical specimens from which <i>M. avium</i> complex isolates used in this study were obtained.	98
3.2.2 Sites of MAC infection/colonisation among the patients in this study.	98
3.2.3 Summary of patients whose strains of <i>M. avium</i> complex were clustered by PFGE of large restriction fragments.	105

List of Figures and Plates

Figures

	Page
1.2.1 Map of the insertion element IS6110 of <i>M. tuberculosis</i> .	28
1.4.1 South East England, showing the location of laboratories which submitted isolates of mycobacteria to Dulwich Public Health Laboratory in 1994.	60
3.1.1 Distribution of bands (IS6110 copies) among <i>M. tuberculosis</i> isolates from 96 HIV seropositive and 84 control patients with tuberculosis.	86
3.1.2 Gelcompar analysis of IS6110 RFLP patterns for <i>M. tuberculosis</i> isolates from HIV seropositive patients.	88
3.1.3 A cluster of cases with indistinguishable strains. Overlap of cases A to C on wards X and Y in hospital 1.	94
3.1.4 Gelcompar analysis of IS6110 RFLP patterns for <i>M. tuberculosis</i> isolates from control patients.	96

Plates

1 Autophotograph of IS6110 RFLP patterns obtained for 22 <i>M. tuberculosis</i> isolates from 18 HIV seropositive patients.	84
2 Autophotograph of internal molecular weight markers.	85
3 A cluster of cases with indistinguishable strains. IS6110 RFLP patterns of cases A to D.	90
4 <i>Ase</i> I large restriction fragments of <i>M. avium</i> complex genomic DNA separated by PFGE. Multiple isolates from five different patients.	100
5 <i>Ase</i> I large restriction fragments of <i>M. avium</i> complex genomic DNA separated by PFGE, showing clusters of cases with similar strains.	103
6 <i>Xba</i> I large restriction fragment patterns of <i>M. avium</i> complex genomic DNA separated by PFGE.	104

1. INTRODUCTION

1.1 MYCOBACTERIAL INFECTIONS IN HIV PATIENTS

Mycobacteria are the organisms most frequently responsible for opportunistic bacterial infections in patients infected with human immunodeficiency viruses (HIV). This is likely to be because effective defence against mycobacterial infection is dependent on the function of activated macrophages. These cells are activated by cytokines including interferon-gamma, produced by T-helper (Th-1) lymphocytes in response to the presentation of mycobacterial antigens. Many changes in the immune system occur as a consequence of HIV infection, including alteration in the balance of endogenous cytokines (Fauci *et al.* 1996), reduced macrophage function, and gradual depletion of the number and function of CD4+ lymphocytes, especially the Th-1 subset. These changes adversely affect the ability to overcome infection by mycobacteria. Furthermore, as the CD4+ lymphocyte count gradually declines, different clinical patterns of mycobacterial infection are encountered (Lucas & Nelson 1994). Thus, when cell mediated immunity is still relatively intact pulmonary tuberculosis may be seen, whereas extrapulmonary disease is more characteristic of later stages. Miliary tuberculosis and disseminated infection with atypical mycobacteria usually occur only when the CD4+ count has fallen to very low levels.

As the total number of HIV seropositive persons has risen since the early 1980s, the isolation of mycobacteria from this group has also increased (Horsburgh & Selik 1989, Yates *et al.* 1993, Benson 1994, Taylor *et al.* 1995). Many species of mycobacteria have been found to cause disease in HIV patients. These include *Mycobacterium kansasii* (Carpenter & Parks 1991, Witzig *et al.* 1995), which may be

responsible for localised pulmonary disease or disseminated infection, *M. xenopi*, *M. fortuitum* (Shafer & Sierra 1992), *M. chelonae*, *M. haemophilum* (Chin 1993), *M. malmoense*, *M. genavense* (Bottger *et al.* 1992, Maier *et al.* 1995) and *M. celatum* (Piersimoni *et al.* 1994). By far the most important, however, are *M. tuberculosis* and *M. avium*. In addition, new patterns in the presentation and epidemiology of mycobacterial infections have become apparent. For example, outbreaks of tuberculosis, often with accelerated progression of disease in those affected and sometimes involving multidrug resistant strains, have been particularly associated with HIV groups; disseminated infection with *M. avium* complex (MAC) is common in patients with acquired immunodeficiency syndrome (AIDS) but extremely rare in other patients.

At the same time that these challenging clinical problems have emerged, new molecular biological techniques have been developed and have been applied to typing the organisms responsible. The results of these studies are giving new insight into the epidemiology of these infections.

1.2 MYCOBACTERIUM TUBERCULOSIS

1.2.1 Trends in tuberculosis notifications and the contribution of HIV co-infection

Tuberculosis, that is, active clinical disease following *M. tuberculosis* infection, remains one of the most common fatal infectious diseases of man. In the last ten years the reversal of the steady decline in tuberculosis seen in the developed and parts of the developing world has been a matter of great concern (Bloom & Murray 1992, Horsburgh & Pozniak 1993, Uttley & Pozniak 1994). The World Health Organisation (WHO) estimated that in 1990 there were eight million new cases of tuberculosis and nearly three million deaths; notifications rose by 24.6% compared to the averages of the mid 1980s (WHO 1992). HIV infection is the most important risk factor for tuberculosis (Styblo & Enarson 1991, Horsburgh & Pozniak 1993, Haas & Des Prez 1994, Schulzer *et al.* 1994). The relative risk of developing tuberculosis following infection with *M. tuberculosis* is 170.3 for patients with AIDS, compared to 30 for those with silicosis and 11.9 for patients on immunosuppressive treatment (A. Pozniak, personal communication). Although its role varies from one part of the world to another, HIV is partly responsible for recent changes in tuberculosis epidemiology.

Developing countries

The association between HIV and the observed increase in tuberculosis is strongest in developing countries. The annual risk of infection with *M. tuberculosis* is highest in

sub-Saharan Africa where the majority of clinical cases are under 50 years of age (Kumar & Watson 1994). This is the same population that is at risk of HIV infection through predominantly heterosexual spread, and studies have shown that the seroprevalence of HIV in patients presenting with clinical tuberculosis is up to 60% (Harries 1990, Elliott *et al.* 1993, van den Broek *et al.* 1993, Houston *et al.* 1994, Richards *et al.* 1995, van Cleeff & Chum 1995). HIV is becoming more common in the Indian subcontinent and Southeast Asia, where a significant proportion of global tuberculosis cases occur. There is evidence of a rising trend of HIV infection in patients with pulmonary tuberculosis in India (Solomon *et al.* 1995) and the potential exists for a crisis in tuberculosis control in that part of the world (WHO 1994).

United States of America

In the United States reported cases of tuberculosis decreased from the 1950s until the mid 1980s. Between 1985 and 1992 however the number increased again by nearly 20%, resulting in an estimated 63000 excess cases over the number expected had the trend from 1980 to 1984 continued (Centers for Disease Control 1993). Much of the increase occurred in young adults living in inner city areas and has been attributed to a large extent to tuberculosis in patients coinfecting with HIV. Analysis of matching cases of tuberculosis and AIDS reported to Centers for Disease Control, Atlanta suggested that at least 30% of the excess cases of tuberculosis during the period 1985-90 are attributable to HIV-induced immunosuppression (Burwen *et al.* 1995).

Although there is evidence of considerable overlap between tuberculosis and HIV in New York City (Greenberg *et al.* 1995), other factors have also played an important part in contributing to the rise: homelessness and overcrowding in inner city areas,

intravenous drug use, increased immigration from countries with a high prevalence of tuberculosis, and a decline in resources for tuberculosis control programmes (Snider & Roper 1992).

Western Europe

Notifications of tuberculosis have continued to decline in some Western European countries, whereas in others they have levelled off or increased, for example in Ireland, Italy and the Netherlands (Raviglione *et al.* 1993). In general HIV appears to have had only a marginal effect on this trend, although it may be more important in the Paris area and among groups of HIV patients in Italy and Spain. A multicentre European study that reviewed 6572 patients diagnosed with AIDS between 1979 and 1989 found that extrapulmonary tuberculosis was much more frequent in Southern Europe than in Central or Northern regions, either at the time of the AIDS diagnosis or at subsequent follow-up (AIDS in Europe, 1996). For other European regions, demographic changes in the populations, with increasing numbers of elderly persons who are liable to reactivation of infection acquired earlier in life, and an increase in the number of immigrants from areas with a high prevalence of tuberculosis have been responsible for these changes (Raviglione *et al.* 1993, Liard *et al.* 1994).

United Kingdom

Although deaths from tuberculosis in England and Wales reported to the Office of Population Censuses and Surveys have continued to decline in the last thirty years, the total number of notifications of tuberculosis has risen slightly since 1987. In that year 5086 cases were notified in England and Wales, compared to 5799 in 1992 and

(provisionally) 6052 in 1993. Notification rates were highest in the elderly, especially in men (Kumar & Watson 1994). The increase is most marked in London, where notified cases of tuberculosis rose from 21 to 31 per 100000 between 1987 and 1994. 37% of all cases in 1994 were in London residents, who make up 13% of the population of England and Wales (Communicable Disease Surveillance Centre 1996a). A survey of notifications carried out in 1988 showed that the rate in immigrants originating from the Indian subcontinent was nearly 25 times that in the white population and accounted for 39% of all notifications (Medical Research Council Cardiothoracic Epidemiology Group 1992). It is unlikely that HIV played a significant role in cases of tuberculosis in Britain during this period because the overlap between those seropositive for HIV, mostly young white men, and those with previous tuberculous infection, mostly the elderly and those from the Indian subcontinent, was small (Watson & Gill 1990, Watson 1993, Watson *et al.* 1993). Such an overlap is mainly limited to immigrants from sub-Saharan Africa. A further survey of notifications of cases of tuberculosis was carried out by the Communicable Disease Surveillance Centre (CDSC) of the Public Health Laboratory Service in collaboration with the British Thoracic Society and Department of Health in 1993. As part of the survey the prevalence of HIV infection was estimated in cases aged 16 to 54 years by unlinked anonymous HIV testing. About 40% of those eligible were tested and the prevalence of HIV infection in this group was approximately 2% (Communicable Disease Surveillance Centre 1994). These preliminary results may be an underestimate of the true prevalence of HIV infection in patients with tuberculosis notified in 1993, but confirm that HIV plays only a small part in the epidemiology of tuberculosis in England and Wales at present. Other factors for the increase are

therefore likely to be involved. Tuberculosis is known to be common among the homeless, whose numbers have increased in recent years. A survey at a temporary shelter for the homeless in London at Christmas 1992 confirmed this to be the case (Kumar *et al.* 1993). However, many patients in this group are unlikely to reach medical attention and to be notified. Another possibility is an increase in cases in recent immigrants to the United Kingdom: the 1993 national survey showed a significant rise in cases among those of black African origin. A review of African AIDS patients in London between 1982 and 1995 found that tuberculosis was much more common among African than non-African patients, accounting for 27% of initial episodes of AIDS in Africans compared to 5% in non-Africans. The incidence of tuberculosis in patients with another AIDS-indicator disease was also higher among Africans: 16 (versus 6) per 100 person-years (Del Amo *et al.* 1996). The median stay in the United Kingdom among African patients in this study from whom information was available was only 12 months. In Hackney, East London, however, tuberculosis in recently arrived immigrants accounted for less than half of the increase in notifications between 1986 and 1993 (Bhatti *et al.* 1995). Demographic changes leading to reactivation of old infection in the increasing elderly population, and an improvement in notification rates, associated with the creation of the role of Consultant in Communicable Disease Control in 1988 are also possible contributing factors. Two recent studies however suggest that the most important contributory factor for the increasing incidence of tuberculosis is socioeconomic deprivation. In England and Wales between 1980 and 1992 an increase occurred only in the poorest 30% of the population and was greatest in the poorest 10% (Bhatti *et al.* 1995, Mangtani *et al.* 1995).

1.2.2 Tuberculosis in patients with HIV infection

Persons infected with HIV are at increased risk of tuberculosis in two ways.

Reactivation of latent tuberculous infection becomes more likely as the number of functioning CD4 lymphocytes is progressively depleted as a result of HIV. For example, in a prospective study of intravenous drug users in New York whose tuberculin sensitivity (an indicator of previous infection with *M. tuberculosis*) and HIV status were known, it was shown that the rate of tuberculosis in the HIV-seropositive cohort was 7.9 per 100 person years in those who were initially tuberculin positive compared to 0.3 per 100 person years in those who were tuberculin negative. The rate of conversion from negative to positive tuberculin sensitivity was similar for both HIV positive and negative subjects during the study (Selwyn *et al.* 1989). A similar relationship between incidence rate of tuberculosis and tuberculin skin test reactivity in Italian HIV infected patients was reported by Antonucci *et al.* (1995), who also demonstrated increasing incidence of tuberculosis with declining CD4 lymphocyte count. Secondly, because of impaired cell-mediated immunity, HIV patients who have not previously been infected are more likely to develop active primary tuberculosis if exposed to tubercle bacilli. Thus, in an outbreak of tuberculosis in a housing facility for HIV patients in San Francisco 15 out of 30 residents exposed to a source were infected and in 11 of these (37%) infection rapidly progressed to active disease. By comparison, none of the staff members exposed to the same source developed active tuberculosis although some had positive tuberculin tests (Daley *et al.* 1992).

Although tuberculosis may occur at any stage during the course of HIV infection it is

more often seen in the later stages (Girardi *et al.* 1994). The likely clinical and radiographic manifestations differ considerably as the CD4 cell count progressively declines. Typical features of post-primary pulmonary tuberculosis such as upper lobe disease with cavitation are more often seen in patients at an early stage of HIV infection when the CD4 count is still high. As immunodeficiency worsens, lower zone infiltration, thoracic lymphadenopathy, as well as extrapulmonary disease and disseminated infection with mycobacteraemia are more common. Conversely, features that result from delayed hypersensitivity such as tuberculous pleural effusion and a positive tuberculin skin test are often absent with low CD4 cell counts (Barnes *et al.* 1991, Barnes *et al.* 1993, Jones *et al.* 1993, Given *et al.* 1994, Post *et al.* 1995, Daley *et al.* 1995). Tuberculosis in patients with HIV-2 infection gives rise to clinical features similar to those seen in HIV-1 co-infection (Gnaore *et al.* 1993). The evidence from developing countries is that patients with HIV and pulmonary tuberculosis are not more infectious to others than seronegative tuberculosis patients (Elliott *et al.* 1993b, Nunn *et al.* 1994).

The interrelationship between HIV and *M. tuberculosis* co-infection may also result in accelerated progression of HIV disease. Experiments in human monocyte cell lines infected with HIV have shown that phagocytosis of *M. tuberculosis* or of its purified protein derivative enhances transcription of integrated provirus genes, although release of intact HIV from such cells may not be increased (Lederman *et al.* 1994, Shattock *et al.* 1994). Similarly, activation of HIV-infected T lymphocytes and macrophages by *M. tuberculosis* enhances release of tumour necrosis factor- α and β_2 -microglobulin, which may lead to increased HIV expression (Wallis *et al.* 1993). Goletti *et al.*

(1996) have demonstrated that active tuberculosis increases HIV replication *in vivo*, as shown by a 5 to 160-fold increase in levels of plasma viraemia during the acute phase of disease which subsequently returns to baseline levels after successful treatment.

These authors also showed that viral replication increased in response to *M.*

tuberculosis in an *in vitro* model, and concluded that activation and infection of CD4+ T cells responding to the mycobacterial challenge are most likely to be responsible for increased viral production.

Two important factors associated with tuberculosis in HIV patients, particularly seen in the United States, are the occurrence of outbreaks of infection in hospitals and other settings and the emergence of multiply drug-resistant strains of *M. tuberculosis*. Nosocomial transmission of tuberculosis on a ward for HIV patients in Verona, Italy was first described by di Perri *et al.* in 1989. Subsequently, many hospital outbreaks have been recognised, especially in Florida and New York (Centers for Disease Control 1991a, Beck-Sague *et al.* 1992, Edlin *et al.* 1992, Pearson *et al.* 1992, Barnes *et al.* 1993, Coronado *et al.* 1993). Transmission of *M. tuberculosis* between HIV patients in hospital may be facilitated when diagnosis and treatment are delayed because of failure to recognise atypical presentations of tuberculosis in this group. In addition, when procedures which induce coughing such as sputum induction, bronchoscopy and administration of nebulised drugs are carried out in conditions with inadequate ventilation the concentration of infectious droplet nuclei in the air is increased. Lack of appropriate isolation facilities for tuberculosis patients in some units may exacerbate the problem (Snider & Roper 1992). Outbreaks of tuberculosis among HIV patients have also occurred in prisons (Valway *et al.* 1994), in residential

facilities (Daley *et al.* 1992), and in shelters for the homeless (Centers for Disease Control 1991b). Transmission to health care workers and to prison staff, who may be seronegative for HIV, is well recognised.

Many of the strains of *M. tuberculosis* identified in these epidemics have been multiply drug resistant (Snider & Roper 1992, Barnes *et al.* 1993, Haas & Des Prez 1994), that is resistant to at least two antituberculous drugs including rifampicin and isoniazid. Strains resistant to up to seven drugs have been isolated. Most modern regimens which are effective for treating susceptible strains employ rifampicin, isoniazid and pyrazinamide. Other antituberculous drugs are often difficult to administer and/or commonly cause serious adverse effects, particularly in AIDS patients. When first recognised, the mortality of multidrug resistant tuberculosis (MDRTB) was between 70 and 90%. In one hospital the median time from diagnosis to death was 2.1 months compared to 14.6 months in HIV seropositive patients with tuberculosis caused by drug sensitive strains (Fischl *et al.* 1992). Delay in recognising infection caused by a multidrug-resistant strain, partly due to the long interval of 4 to 11 weeks required for primary isolation of the organism from a clinical specimen followed by susceptibility testing by conventional methods, contributes to the high mortality of this infection. Furthermore, patients treated with an inappropriate regimen until susceptibility results are available may continue to transmit the resistant strain to others. More recent reports however, suggest that improved clinical outcomes for patients with MDRTB can be achieved, particularly if disease is recognised early and at least two drugs to which the initial isolate is susceptible *in vitro* are promptly administered (Salomon *et al.* 1995, Turett *et al.*

1995).

Great efforts have been made to control the spread of MDRTB among HIV patients and healthcare workers in the United States. Guidelines for limiting spread of airborne pathogens published by the Centers for Disease Control (CDC) in 1990 and 1994 include recommendations for negative pressure ventilation, high efficiency particulate air filtration, germicidal UVA irradiation of the air supply and use of high efficiency respirators by staff. These measures are expensive to implement, and respirators in particular are unpopular with healthcare workers and of doubtful cost-effectiveness (Adal *et al.* 1994). Nevertheless, effective control of nosocomial transmission of MDRTB has been demonstrated in a Florida hospital by implementation of these measures (Wenger *et al.* 1995). Transmission of MDRTB among AIDS patients in a hospital in New York City also decreased markedly when CDC guidelines were implemented (Stroud *et al.* 1995). The greatest change was observed after enforcement of simple administrative measures, such as isolating patients with suspected tuberculosis in side rooms, before engineering changes (improved ventilation to the rooms) were made. Infection among staff, as judged by tuberculin skin test conversion rate also fell when CDC guidelines were put into practice at a New York hospital. The decrease, from 20.7% to 5.8% between 1991 and 1993, occurred before the introduction of high efficiency particulate air filter respirators (Fella *et al.* 1995).

It is likely that MDRTB originated under the selective pressure of inadequate chemotherapy because of inappropriate selection of therapy and poor compliance by

some patients (Riley 1993). In New York City where most cases of MDRTB have occurred, dismantling of an effective tuberculosis control program (chest clinics, patient home visits, etc.), non-compliance with medication (86% of patients starting antituberculous therapy were lost to follow-up or failed to complete therapy in one study (Brudney & Dobkin 1991)), worsening socioeconomic conditions such as homelessness, and HIV infection have all contributed to this major public health problem. The restoration of tuberculosis control programs, and especially use of directly observed therapy, is helping to reverse the trend (CDC 1995). Evaluation of suitable drug regimens for patients with multidrug-resistant strains (Iseman 1993), and the development of more rapid methods for the detection of *M. tuberculosis* in clinical specimens and for susceptibility testing, including use of molecular biological methods (Drobniewski *et al.* 1994), will facilitate the management of patients with MDRTB.

1.2.3 Typing methods for *Mycobacterium tuberculosis*

The study of tuberculosis epidemiology has been greatly enhanced in recent years by the development of molecular methods of typing *M. tuberculosis* which are capable of typing nearly all strains and of reproducibly distinguishing between them with great discriminative power. Such methods allow outbreaks of tuberculosis to be traced precisely and on a larger scale may be applied to cases in populations in order to monitor patterns of disease transmission, for example the relative contributions of exogenous infection and reactivation.

Previously, methods based on phenotype were used with limited success in an attempt to distinguish different isolates of *M. tuberculosis*. Phage typing was available but little used because of limited discriminative power, technical difficulty and poor reproducibility between laboratories (Rado *et al.* 1975). Larger numbers of phages would be required to improve discriminative power but standardisation of phage stocks remains a problem. Multilocus enzyme electrophoresis has also been applied to mycobacteria, but the method was technically demanding and found members of the *M. tuberculosis* complex to be genetically homogeneous, supporting the concept of a single species (Wasem *et al.* 1981, Feizabadi *et al.* 1996). High performance liquid chromatography which analyses mycolic acids of the mycobacterial cell wall is able to distinguish different species of mycobacteria and to some extent between strains of *M. tuberculosis* (Butler *et al.* 1991). Again, its limited discriminative power is not good enough for epidemiological purposes. Alternatives to methods based on phenotype are therefore required.

Restriction fragment length polymorphism analysis

Genotypic methods of distinguishing strains of *M. tuberculosis* employ restriction endonucleases which cut chromosomal DNA at specific sites to exploit slight differences in DNA sequences between strains that do not give rise to differences in phenotype. Restriction fragments thus produced may be separated by gel electrophoresis and visualised directly by ethidium bromide staining. This simple approach is not very useful for *M. tuberculosis* however, as the patterns produced are very complex and relatively homogeneous (Shoemaker *et al.* 1986). Better results have been obtained when high molecular weight fragments, produced with restriction

endonucleases that cut genomic DNA infrequently, are separated by pulsed-field gel electrophoresis (see section 1.3.6). In this way Zhang *et al.* (1992) were able to separate 26 isolates of *M. tuberculosis* into 10 clusters of related isolates. The isolates within each cluster yielded indistinguishable large restriction fragment patterns and the grouping into clusters was confirmed by hybridisation with a labelled probe to IS6110.

An alternative technique for analysis of the variation in the position of the restriction enzyme cutting sites (restriction fragment length polymorphism, RFLP) is to transfer the DNA fragments after electrophoresis to a suitable membrane by Southern blotting and then to hybridise with a labelled probe of a selected repetitive element. This allows detection of the fragments of different sizes which possess copies of the appropriate repetitive element. A characteristic RFLP pattern or 'fingerprint' is generated for each strain. Probes to ribosomal RNA genes have been used for many bacterial species since such genes are usually present in multiple copies and their sequence is highly conserved. However, ribotyping is unsuitable for *M. tuberculosis* since this species has only a single copy of rRNA genes which would give inadequate polymorphism.

Insertion sequence IS6110 and its role in studies of molecular epidemiology

Insertion sequences are suitable repetitive elements for use as probes in RFLP analysis because they are usually present in multiple copies and are mobile throughout the genomic DNA to a greater or lesser extent giving rise to polymorphism of position. They characteristically contain short inverted terminal repeat sequences and genes

coding for proteins involved in transposition. The insertion sequence IS6110 has been the one used most widely in studies of *M. tuberculosis* to date. IS6110 was sequenced by Thierry *et al.* (1990a,b) and shown to be related to the IS3 family of insertion sequences found in the *Enterobacteriaceae*. Two other insertion sequences, IS986, isolated from a *M. fortuitum* plasmid (Zainuddin & Dale 1989, McAdam *et al.* 1990), and IS987 from a strain of *M. bovis* BCG (Hermans *et al.* 1991), have sequences which differ from IS6110 by only a few base pairs and can be considered as essentially the same element. IS6110 is 1355 base pairs in length and contains a single restriction site for *Pvu* II at base pair 461 (Figure 1.2.1). It is very well suited to typing studies of *M. tuberculosis* because it is confined to the *M. tuberculosis* complex, is present in almost all strains usually in multiple copies, and is distributed throughout the genome with considerable polymorphism between strains. At the same time, the location of IS6110 in the genomes of different strains is very stable: repeated *in vitro* cultures or animal passages do not generally result in changes in its distribution (van Soolingen *et al.* 1991). Fingerprint patterns of repeated isolates recovered from individual patients during intervals of up to four and a half years remain identical or virtually identical (Otal *et al.* 1991, Cave *et al.* 1994). A standardised methodology for RFLP analysis of *M. tuberculosis* has been proposed to facilitate comparison of results between different groups. This uses the restriction endonuclease *Pvu* II and a DNA probe which hybridises to a fragment of IS6110 to the right of this restriction site (van Embden *et al.* 1993).

This method has been extremely valuable in epidemiological investigations of outbreaks of tuberculosis: isolates from epidemiologically linked cases have

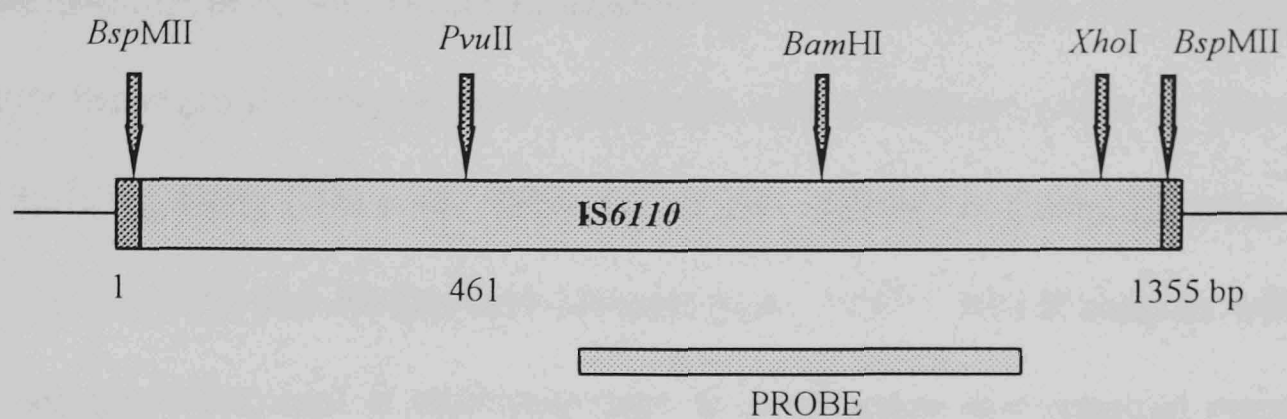


Figure 1.2.1

Map of the insertion element IS6110 of *M. tuberculosis*

The insertion element is 1355 base pairs in length, and is bordered by 28 base pair inverted repeats (shaded). The cleavage sites of several restriction enzymes are shown, including *Pvu* II which cleaves at base pair 461. A DNA probe to the right of this restriction site as shown is used in the standardised methodology for RFLP analysis of *M. tuberculosis*.

Based on van Embden *et al.* (1993).

indistinguishable or very closely related banding patterns, whereas unrelated isolates show great diversity (Hermans *et al.* 1990, CDC 1991, van Soolingen *et al.* 1991, Beck-Sague *et al.* 1992, Daley *et al.* 1992, Edlin *et al.* 1992, Godfrey-Faussett *et al.* 1992, Coronado *et al.* 1993, Valway *et al.* 1994). When multiple isolates are available from patients with recurrent tuberculosis it is also possible to distinguish relapse of the original infection from reinfection with a different strain, as shown by studies in Hong Kong (Das *et al.* 1993) and of HIV patients in Kenya (Godfrey-Faussett *et al.* 1994) and Switzerland (Strassle *et al.* 1997). RFLP analysis with IS6110 has also been used in individual cases to demonstrate that acquired resistance to rifampicin in *M. tuberculosis* does not modify the DNA fingerprint (Godfrey-Faussett *et al.* 1993, Strassle *et al.* 1997).

An important application of RFLP analysis using IS6110 is to the study of tuberculosis epidemiology in large communities. Typing all isolates from each confirmed case of tuberculosis in a defined area over a period of time allows an assessment of the degree of heterogeneity of the strains causing disease in that area. It is believed that the degree of heterogeneity reflects the relative proportions of cases that are due to recent transmission of *M. tuberculosis* or to relapse of old previously acquired infection. Such studies may also disclose previously unrecognised outbreaks of infection in the community (Table 1.2.1).

Less heterogeneity among strains is expected in countries with high rates of tuberculosis or when tuberculosis has only been in a community for a short time. In such circumstances most cases follow recent transmission of the organism. This was

Table 1.2.1 Summary of published community studies of tuberculosis epidemiology using IS6110 RFLP analysis (1991-95).

Study	Region	Main findings
van Soolingen <i>et al</i> 1991	Africa, Netherlands	Less polymorphism among African strains than those from the Netherlands; inversely related to rate of TB in the community
Godfrey-Fausset & Stoker 1992	Malawi, Kenya	18 pairs of isolates among 92 patients
Yang <i>et al</i> 1995	Tanzania	101 different strains among 134 patients. No difference between HIV positive and negative patients.
Chevrel-Dellagi <i>et al</i> 1993	North Tunisia	Much less heterogeneity of strains in one district compared to 3 others. Degree of polymorphism is related to the stability of the population.
Alland <i>et al</i> 1994 Small <i>et al</i> 1994	New York City San Francisco	Up to 40% of cases are part of a cluster. HIV infection, poverty, infection with a drug resistant strain increase chance of being in a cluster. Few clusters were recognised by conventional contact tracing.
Tabet <i>et al</i> 1995	King County, Washington	Community outbreak among 6 HIV patients, not recognised by contact tracing.
Genewein <i>et al</i> 1993	Berne, Switzerland	45 out of 163 patients were potentially linked. Complex network of transmission, largely between native young adults. Only apparent from RFLP analysis of strains.
Yang <i>et al</i> 1995	Denmark	Despite low endemicity of TB, recently transmitted infections comprise at least a quarter of all cases. Chains of transmission in specific regions were recognised.
Torrea <i>et al</i> 1995	French Polynesia	Significant active transmission (11 clusters among 64 patients) occurred in a stable population with a low incidence of TB.

confirmed by van Soolingen *et al.* (1991) who showed a lesser degree of polymorphism among African strains than in those collected in the Netherlands. Another study in Malawi and Kenya (Godfrey-Faussett & Stoker 1992) demonstrated 18 matched pairs of isolates from 92 patients, although no epidemiological links between patients were found. It was nevertheless concluded that heterogeneity amongst strains was sufficient to allow valid molecular epidemiological study of tuberculosis in Africa. Similarly, in Tanzania, a country with high rates of both tuberculosis and HIV, 101 different IS6110 fingerprint patterns were observed among 134 patients with tuberculosis, half of whom also had HIV infection (Yang *et al.* 1995b). The level of diversity in HIV seropositive and negative groups was comparable, suggesting, in this population, an equal risk of infection with a defined *M. tuberculosis* clone regardless of HIV status. However, patterns of tuberculosis transmission appear more complex when examined at a local level. For example, four districts in North Tunisia gave contrasting results when *M. tuberculosis* strains isolated over a three year period were examined (Chevrel-Dellagi *et al.* 1993). Strains from three districts displayed a high degree of polymorphism whereas those from the district of Menzel Bourguiba were much less heterogeneous, with 58% of cases in that area traceable to four *M. tuberculosis* strains. It was suggested that the degree of polymorphism may be related to the stability of the population: higher diversity is expected in large cities or regions with mixed populations because strains are imported from different geographical areas.

In the United States it was assumed until recently that about 90% of active cases of tuberculosis resulted from reactivation of previously acquired infection (Hamburg &

Frieden 1994). The results of typing studies from New York City (Alland *et al.* 1994) and San Francisco (Small *et al.* 1994) however have challenged this view. In the former, 104 patients treated for tuberculosis at a Bronx hospital between 1989 and 1992 had complete medical records and isolates whose RFLP patterns were evaluable. 37.5% of these had patterns identical to at least one other patient; overall, 12 clusters of cases were recognised. The San Francisco study examined the RFLP patterns of strains from patients reported to that city's tuberculosis registry in 1991-92. 191 (40%) of 473 patients evaluated belonged to one of 44 clusters of identical patterns, which were between 2 and 30 persons in size. However, conventional contact tracing only identified links among ten percent of the patients, illustrating the power of this molecular epidemiological tool. In both studies it was assumed that clusters of cases with similar strains result from recent transmission of infection. Risk factors for belonging to a cluster were found to be HIV infection, black race or Hispanic ethnicity (socioeconomic factors rather than genetic predisposition may be more relevant) and infection with a drug resistant strain (as such patients are usually infectious to others for longer). In contrast, older patients and those born abroad were less likely to belong to a cluster, suggesting that reactivation of old infection was the cause of their disease. It is surprising however, that the proportion of cases due to recent transmission was similar in the two cities, when the proportion of patients completing therapy at that time was much higher in San Francisco than in New York City (Hamburg & Frieden 1994).

The strong link between HIV and recently transmitted infection, (nearly two thirds of HIV seropositive patients were part of a cluster), was also found in a study of

community-acquired tuberculosis in King County, Washington, USA (Tabet *et al.* 1994). RFLP analysis on isolates from 18 HIV positive patients and 10 without risk factors for HIV revealed a community outbreak of tuberculosis affecting 6 HIV positive patients whose only common exposure was contact at one or more of three bars. Again, this outbreak was revealed only when RFLP analysis on stored strains of *M. tuberculosis* was performed and had not previously been detected by conventional contact tracing.

Evidence that tuberculosis is spreading in parts of Europe in ways similar to those seen in the United States was obtained from a population study in Berne, Switzerland, in which all isolates from culture positive cases during 1991-92 were examined by RFLP analysis with an IS6110 probe (Genewein *et al.* 1993). 45 of the 163 patients were potentially linked on this basis. Epidemiological investigation uncovered a group of 22 patients which included drug addicts, alcoholics and homeless persons from whom tuberculosis spread to the general population. A complex network pattern of transmission was observed which was not identifiable by conventional contact tracing. Transmission in the native population occurred largely between young adults, whereas tuberculosis in immigrants, although responsible for a large proportion of cases, did not spread to the general population. Furthermore, the group in which tuberculosis was mainly transmitted - i.v. drug users, HIV positive persons and the homeless - is the one most susceptible to the development of drug resistance because of poor compliance with therapy. Multidrug resistance was observed to develop in one case in this study.

Community studies on RFLP analysis of *M. tuberculosis* in other countries with a low incidence of tuberculosis have also revealed unexpectedly high proportions of cases due to recent transmission of infection. In Denmark, a country with a well established tuberculosis control programme and low endemicity of tuberculosis, recently transmitted infections comprised at least a quarter of all cases in 1992, and chains of transmission in specific geographical regions were identified (Yang *et al.* 1995a). However, no data were available on the HIV status of patients involved in these clusters, as the collection of such information is restricted by Danish law. This study also showed that while nearly half of the cases of tuberculosis involved patients of foreign origin, most of these had unique RFLP patterns and were rarely part of active chains of transmission. The authors suggested that clinical suspicion of tuberculosis in native Danish patients with pulmonary symptoms is lower than for immigrants, so that delay in diagnosis and treatment in the former may lead to further transmission of infection. A study in French Polynesia (Torrea *et al.* 1995), where there is a low incidence of tuberculosis in a geographically stable population, has also suggested that active transmission of infection occurs to a significant extent in that community. 38 different IS6110 types were found among isolates from 64 patients which clustered in 11 groups.

As outlined above, RFLP analysis using IS6110 has been the most widely used molecular typing method for *M. tuberculosis* to date and has greatly contributed to our understanding of the epidemiology of infection caused by this organism (Hayward 1995). A limitation of the method, however, is its restricted ability to distinguish between the minority of strains which carry only one or two copies of IS6110.

Strains with only one copy are more common in certain populations, including those from South India, where they accounted for the isolates from 40% of patients (Das *et al.* 1995), Vietnam (Yuen *et al.* 1993), Malaysia, Tanzania and Oman (Fomukong *et al.* 1994). As in most strains of *M. bovis* BCG, which usually have a single copy of IS6110, the insertion sequence appears to be located in a specific region of the chromosome which may represent a 'hot spot' for IS6110 integration (Hermans *et al.* 1991, van Soolingen *et al.* 1993a). An alternative explanation put forward by Fomukong *et al.* (1994) is that IS6110 may be defective in transposition in these strains and the loss of transposability may have occurred at an early stage in the evolution of the *M. tuberculosis* complex.

Recently, a 267-nucleotide genomic region which is able to carry IS6110 at six alternative locations (the IS6110 preferential locus, *ipl*) has been described (Fang & Forbes 1997). As these authors point out, if IS6110 insertion occurs in such preferred regions of the chromosome rather than at random, then the variety of RFLP patterns produced will be diminished, so that the discriminatory power of this typing method is inversely proportional to the ratio of preferred to random IS6110 insertion sites.

Rarely, strains of confirmed *M. tuberculosis* have been encountered from India and Vietnam that lack IS6110 altogether (van Soolingen *et al.* 1993a, Yuen *et al.* 1993, Das *et al.* 1995). IS6110 RFLP analysis is therefore unsuitable as a tool for epidemiological studies in areas where such strains are prevalent. These strains would also give false negative results in diagnostic tests for *M. tuberculosis* in clinical specimens based on polymerase chain reaction amplification and DNA hybridisation

methods directed towards IS6110.

Other repetitive DNA elements

Other repetitive elements have been evaluated as potential targets for genotypic typing methods (Table 1.2.2).

Table 1.2.2 Repetitive DNA elements in *M. tuberculosis*

Element	Size (bp)	Host range	Copy no.	Suitability for RFLP analysis
IS6110	1355	MTBC	0-25	Widely used
IS1081	1324	MTBC, <i>M. xenopi</i>	5-7	Insufficient polymorphism
MPTR	10	MTBC, <i>M. kansasii</i> , <i>M. goodii</i> , <i>M. szulgae</i> , etc.	> 100	Low discrimination Not often used
PGRS	30	MTBC	> 100	Highly discriminatory Very complex patterns
DR	36	MTBC	10-50	Useful to distinguish strains with similar low copy IS6110 patterns

Abbreviations: MPTR major polymorphic tandem repeat
PGRS polymorphic GC-rich repetitive sequence
DR direct repeat sequence
MTBC *M. tuberculosis* complex

Data from Small & van Embden (1994) and van Soolingen *et al.* (1993a).

IS1081 is a 1324 base pair insertion sequence originally identified in the genome of *M. bovis* (Collins & Stephens 1991) which is virtually exclusive to members of the *M. tuberculosis* complex (van Soolingen *et al.* 1992). Most strains carry five or six copies of IS1081 but unfortunately the sites of insertion of these appear to be highly conserved giving rise to very limited polymorphism between different strains, so that this insertion sequence is not suitable for use in epidemiological investigations (van Soolingen *et al.* 1992, Collins *et al.* 1993). It may however be possible to distinguish *M. bovis* BCG from other *M. tuberculosis* complex strains by the presence in the former of an 8.0 kilobase IS1081-containing *Pvu* II fragment (van Soolingen *et al.* 1992).

Several repetitive DNA elements which are not insertion sequences have also been investigated with regard to potential use in typing. These are (i) the major polymorphic tandem repeat sequences, consisting of a tandemly-repeated unit of ten base pairs with copies separated by five base pair unique sequences (Hermans *et al.* 1992), (ii) a polymorphic GC-rich repetitive sequence (Ross *et al.* 1992), and (iii) the direct repeat sequences, which are 36 base pair sequences found in multiple copies at the 'hot spot' for IS6110 integration into the chromosome (Hermans *et al.* 1991). A study of strains containing only one copy of IS6110, or two copies at the same chromosomal locus, found that RFLP patterns obtained with a probe to the major polymorphic tandem repeat on *Alu* I digested DNA gave insufficient discrimination to be useful. Hybridisation of a probe to the GC-rich repetitive sequence carried on plasmid pTBN12 with the same DNA samples gave highly discriminatory results but the patterns obtained were complex and difficult to interpret. The best results were

found with a probe to the 36 base pair direct repeat region on *Alu* I digested DNA, which was able to distinguish most of the strains that had very similar IS6110 RFLP patterns (van Soolingen *et al.* 1993). This method, direct repeat (DR) RFLP analysis, was successfully used in the molecular epidemiological study of tuberculosis in New York (Alland *et al.* 1994) to sort strains with two identical bands on hybridisation with IS6110 into three smaller clusters. In the French Polynesian study (Torrea *et al.* 1995) DR RFLP was able to confirm the relationship between strains with similar IS6110 RFLP types and also showed two clusters of strains with different IS6110 patterns and the same DR RFLP type, suggesting that the two clusters may have shared a common origin and evolved to new IS6110 types. The combination of RFLP analysis with probes to IS6110 and to the DR region may prove to have even greater value in tracing the spread of *M. tuberculosis* through communities than the use of IS6110 alone. The conclusion that additional genotyping markers are required to enhance accuracy in epidemiological work was also reached in a study of highly polymorphic genomic domains of *M. tuberculosis* which hybridise to the oligonucleotide (GTG)₅ (Warren *et al.* 1996). Probes to these genomic domains were used to genotype strains of *M. tuberculosis* with indistinguishable IS6110 fingerprints isolated in a South African community. The method was able to subclassify IS6110 clusters, including those with a high copy number of IS6110, into smaller clusters and unique strains. Significantly, the molecular data correlated with patient interviews and clinical records, as contacts were only established within subclusters (Warren *et al.* 1996).

Application of the polymerase chain reaction to typing

A significant disadvantage of typing methods based on RFLP analysis is that a relatively large quantity (approximately 0.5 to 1.0 μg per strain) of high quality DNA is required. Subculture of *M. tuberculosis* may take several weeks to generate sufficient cells for DNA extraction. While this delay may be acceptable for a retrospective epidemiological study, assuming that stored strains are viable, it is undesirable where investigation of a continuing outbreak is concerned, particularly if multidrug resistant strains are involved. The RFLP procedure itself entails DNA extraction, restriction endonuclease digestion, electrophoresis and Southern blot hybridisation, and is labour intensive. Several methods based on a polymerase chain reaction (PCR) to amplify small quantities of DNA have been described, which are rapid and require only small numbers of cells.

Ross and Dwyer (1993) developed a PCR typing method for *M. tuberculosis* using oligonucleotide primers to the ends of the IS6110 insertion sequence in order to amplify DNA between copies of this element on the chromosome. They were able to group together strains isolated from epidemiologically related patients with tuberculosis and to distinguish strains from different clusters of cases, but variations in some of the minor PCR products occurred which makes the method less suitable for comparison of large numbers of isolates. A modification of the method, using only one primer, has been used as a screening method for rapid differentiation of clinical *M. tuberculosis* isolates directly from BACTEC broth cultures (Otal *et al.* 1997). Results were consistent with those of the standardised RFLP method although the degree of discrimination was lower.

An alternative method, termed mixed-linker PCR (Haas *et al.* 1993), amplifies fragments of DNA between IS6110 insertion sequences and linkers ligated into *Hha* I restricted genomic DNA at the ends of restriction fragments. This method was used in the investigation of an outbreak of tuberculosis in a renal unit and gave results very similar to those of conventional IS6110 RFLP analysis (Jereb *et al.* 1993). However, careful optimisation of the intermolecular ligation step is required to achieve reproducibility of this complex procedure (Patel *et al.* 1996). A modification of the method with fluorescent detection of PCR products enables DNA fingerprinting analysis to be automated (Butler *et al.* 1996). A slightly different strategy, termed heminested inverse PCR, involves the digestion of mycobacterial DNA with *Bse* FI, circularisation of the resulting restriction fragments, then two sequential polymerase chain reactions which amplify the 5' end of IS6110 together with its upstream flanking sequence (Patel *et al.* 1996). The method is claimed to be suitable for rapid fingerprinting of *M. tuberculosis* strains in epidemiological studies because it requires much less DNA than conventional RFLP analysis, but has an equivalent discriminatory power and is technically simple and reproducible. However, although heminested inverse PCR may be more discriminating than standard RFLP analysis for strains carrying single copies of IS6110, it suffers from a progressive reduction in clarity of banding patterns with increasing IS6110 copy number (Patel *et al.* 1996).

Other repetitive elements in *M. tuberculosis* have been investigated as potential targets for PCR-based typing methods. Double repetitive element PCR, involving amplification of DNA segments located between IS6110 and the polymorphic GC-rich repetitive sequence (Friedman *et al.* 1995), has a discriminatory power similar to that

of IS6110 RFLP analysis, and uses a boiled suspension of bacterial cells as DNA source. It may be suitable as a rapid screening method in epidemiological investigations to subdivide a large number of isolates into clusters for further typing by RFLP analysis. An alternative method, which can generate reliable patterns directly from sputum specimens as well as from cultures, uses a PCR and hybridisation procedure to measure the variability among strains of the distances between IS6110 elements and copies of the major polymorphic tandem repeat sequence (Plikaytis *et al.* 1993). Another approach is a PCR-based fingerprinting method using the (GTG)₅ oligonucleotide as a primer in association with an IS6110 primer. This was found to be more discriminating than traditional IS6110 typing in a study of isolates from different regions of Italy and Pakistan (Sechi *et al.* 1996).

Each of these techniques uses at least one primer derived from the structure of IS6110, so that the pattern of the PCR products obtained is not independent of the number and distribution of IS6110 copies in the genome. Two typing methods exploit the DNA polymorphism in the spacers found at the direct repeat region of the chromosome in *M. tuberculosis* complex strains. Direct variable repeat PCR gives the sequence of the spacer residues between each 36 base pair direct repeat according to the first residue at the 5' end of each spacer (Groenen *et al.* 1993). 'Spoligotyping' amplifies the spacers present in a strain by PCR using primers which anneal at each end of the direct repeat, then detects the presence or absence of spacers of known sequence by hybridisation of the biotin-labelled PCR product to known spacer oligonucleotides immobilised on a membrane (Kamerbeek *et al.* 1994).

Two technical problems with the use of PCR for fingerprinting have been raised (Saunders & Ridley 1994). Amplification of large template molecules is less efficient than that of small fragments and some may not be reliably detected. Methods based on amplification of DNA between adjacent copies of *IS6110* (Ross & Dwyer 1993), or between *IS6110* and another repetitive element (Plikaytis *et al.* 1993) depend on the repetitive elements (and hence priming sites) being close enough together for efficient PCR. In practice, few amplicons are often produced, limiting the discriminative power of the methods (Patel *et al.* 1996). Secondly, partial hybrid structures may be produced in the later cycles of PCR when multiple templates with limited sequence homology are amplified together, which may appear as artefacts. Further work to assess the reproducibility and discriminatory power of these techniques is therefore required.

Random amplified polymorphic DNA analysis (RAPD) is a variant of the polymerase chain reaction which has been used successfully to type a wide variety of microorganisms. It does not require genetic knowledge of the target organism but instead uses a single arbitrary primer which binds to low stringency priming sites on both strands of DNA close enough together for PCR amplification to occur (Matthews 1993). This method has been applied to *M. tuberculosis* and was found to be a rapid and simple alternative to RFLP analysis (Linton *et al.* 1994). However, many primers had to be tested before optimal results were obtained, and inconsistency of the banding pattern between experiments is a particular problem with RAPD. A modified method has been reported (Abed *et al.* 1995) in which PCR products obtained by amplifying the region between 16S and 23S ribosomal RNA genes are used as target

DNA for RAPD. This generates patterns which are easier to read and have greater discriminatory power compared to use of whole genomic DNA as RAPD template. Reproducibility of these methods between laboratories remains to be established however.

1.3 *MYCOBACTERIUM AVIUM* COMPLEX

1.3.1 Infections caused by *M. avium* complex

M. avium complex (MAC) is increasingly recognised as an important opportunistic pathogen in patients with AIDS. This organism is an uncommon cause of chronic pulmonary infection in adults, usually those with pre-existing lung disease (Wolinsky 1979), and in children primary infection may result in cervical lymphadenitis (Wolinsky 1995). Disseminated infection is virtually confined to patients with very low CD4 lymphocyte counts (<100 per μl) as a result of HIV infection (Ellner *et al.* 1991, Benson & Ellner 1993, Inderlied *et al.* 1993, Falkinham 1994). Up to 53% of AIDS patients in the United States on whom autopsies are carried out have evidence of disseminated MAC infection (Hawkins *et al.* 1986), making it one of the most frequent infective complications of AIDS in the Western world. Of over 12 000 cases of disseminated non-tuberculous mycobacterial infection in AIDS patients reported to the Centers for Disease Control, Atlanta by December 1990, over 96% were due to MAC (Horsburgh 1991). This infection is also frequently seen in AIDS patients in Europe and Australia, although it appears to be uncommon in Africa (Benson & Ellner 1993, Pozniak *et al.* 1996). In the West, the incidence of disseminated MAC infection has increased in recent years (Nassos *et al.* 1991, Yates *et al.* 1993). This may be due to the increasing number of HIV patients who survive to reach very low CD4 counts as a result of anti-retroviral drug therapy and effective prophylaxis and treatment of other opportunistic infections. The increase may also partly reflect a greater enthusiasm by clinicians to seek and confirm MAC infection as its contribution

to morbidity and mortality in AIDS is more widely acknowledged (Jacobson *et al.* 1991, Chin *et al.* 1994c), and as simple blood culture techniques for diagnosis and relatively non-toxic antimicrobials for treatment are available (Ellner *et al.* 1991, Benson & Ellner 1993).

1.3.2 Microbiology of the *M. avium* complex

Organisms of the *M. avium* complex are slow-growing acid fast bacilli which are usually non-pigmented although some strains may produce a yellow pigment in the absence of light (Inderlied *et al.* 1993). They belong to the species *M. avium* and *M. intracellulare*; *M. scrofulaceum* is related but no longer included in the complex. Recently, *M. celatum*, which closely resembles *M. avium*, has also been recognised as a cause of disseminated infection in AIDS patients (Piersimoni *et al.* 1994). On the basis of phenotypic properties and nucleic acid studies three subspecies of *M. avium* have been proposed: *M. avium* subsp. *avium*, *M. avium* subsp. *paratuberculosis* and *M. avium* subsp. *silvaticum* (Thorel *et al.* 1990). Members of the group may be distinguished by serological specificities conferred by oligosaccharide residues of the C-mycoside glycopeptidolipids which are integral constituents of the cell wall and envelope. 28 serovars are known, but the ones most often responsible for disseminated infection in AIDS patients are serovars 1, 4 and 8, all belonging to *M. avium* (Inderlied *et al.* 1993).

1.3.3 Environmental sources and pathogenesis

To date, there has been no evidence of person to person spread of MAC, and the source of infection in most cases is presumed to be the environment. MAC is widely distributed and has been isolated from soil, bird faeces, food, cigarettes, natural water sources and domestic and hospital piped water supplies (du Moulin *et al.* 1988, Eaton *et al.* 1995, Peters *et al.* 1995, Yajko *et al.* 1995, Pozniak *et al.* 1996). The frequency with which it is isolated from soil samples varies from one geographical area to another, but does not necessarily correlate with the frequency of disease due to MAC in the same areas. For example, disseminated MAC infection is more common in the north-east than in the south-east United States, although the likelihood of isolating MAC from the environment shows the opposite trend (Falkinham *et al.* 1980). Likewise, in African AIDS patients disseminated MAC infection is rare, although high numbers of MAC have been recovered from soil and water samples in Zaire, Uganda and Kenya (von Reyn *et al.* 1993b). A study of the home environment of AIDS patients in California (Yajko *et al.* 1995) suggested that the soil of potted plants rather than water or food may be a significant reservoir of *M. avium* complex. However, potted plants are usually watered with tapwater and the potting compost is likely to be more favourable to the survival and multiplication of MAC than treated piped water, so this finding does not exclude water as the original source of MAC.

The pathogenesis of disseminated MAC infection has not been definitely established, but it is likely that in most cases recent acquisition of the organism rather than reactivation of latent infection occurs and that the portal of entry to the body is

usually the gastrointestinal tract or the respiratory tract. This is supported by observations that skin test reactivity against *M. avium* antigens is infrequent amongst healthy individuals (von Reyn *et al.* 1993a), and that in some AIDS patients gastrointestinal or respiratory colonisation may be detected before the onset of disseminated disease (Damsker & Bottone 1985, Hellyer *et al.* 1993, Chin *et al.* 1994a, Havlik *et al.* 1994). Thus in a prospective study of HIV patients with CD4 counts of ≤ 50 cells/ μ l the risk of MAC bacteraemia within one year was 60% for patients with colonisation of either respiratory or gastrointestinal tract and was greater than for those without MAC in these sites (Chin *et al.* 1994a). Of these two sites the gut may more often be the portal of entry of MAC: it is more often isolated from the gut than the respiratory tract in HIV patients, and the frequency of gastrointestinal colonisation increases with the progression of HIV disease (Havlik *et al.* 1994). Recently, a cluster of cases associated with a hospital water supply provides further support to the hypothesis that disseminated MAC infection follows recent acquisition of the organism (von Reyn *et al.* 1994). Aerosols generated from natural water sources have been found to concentrate MAC (Wendt *et al.* 1980) and may consist of droplets sufficiently small to reach the alveoli, but whether inhalation of such aerosols or ingestion of the water is more often the route of MAC colonisation is unknown. However, in a case-control study of possible environmental risk factors for acquisition of MAC daily showering, a procedure expected to generate aerosols from the hot water supply, was actually shown to be protective (Horsburgh *et al.* 1994). The same study also found that consumption of hard cheese was a risk factor for disseminated disease but the association may have been spurious; MAC was not isolated from cultures of cheese (Yajko *et al.* 1995, Horsburgh *et al.* 1994).

Although colonisation of the gastrointestinal or respiratory tract may precede disseminated infection with MAC in many cases, prospective studies have shown that it is not always possible to detect the colonisation stage by means of routine cultures, and conversely, some patients with positive cultures do not progress to dissemination, so such surveillance has limited predictive value for disseminated disease (Havlik *et al.* 1994). The latter occurs virtually exclusively in patients with severely depressed CD4 cell counts; the risk of MAC bacteraemia rises progressively as the CD4 count falls (Nightingale *et al.* 1992). Persistent bacteraemia and heavy tissue loads of organisms, up to 10^9 to 10^{10} colony forming units per gram may be found (Wong *et al.* 1985). Organisms are present predominantly in the mesenteric lymph nodes and Peyer's patches of the gastrointestinal tract and in the liver, spleen and bone marrow, although almost any organ may be found to be involved at autopsy. They are usually present within macrophages, with little evidence of granuloma formation or inflammatory response (Bermudez *et al.* 1992, Benson & Ellner 1993, Chin 1993). Phagocytosis of MAC by macrophages appears to be unimpaired, but intracellular killing of the organisms does not occur. Production and release of cytokines including tumour necrosis factor and GM-CSF is reduced in MAC-infected macrophages. Not all patients who have MAC cultured from blood have unremitting bacteraemia: in a small proportion bacteraemia is transient, becoming undetectable in the absence of antimycobacterial therapy. Symptoms are generally fewer and survival longer in this group (Kemper *et al.* 1994).

1.3.4 Virulence factors

Characteristics of MAC strains that correlate with virulence have been sought. Strains isolated from AIDS patients multiply more rapidly in the beige mouse model of disseminated MAC infection than strains isolated from the environment. Such strains form mostly smooth flat transparent colonies on Middlebrook 7H11 agar, compared to environmental strains which more often give rise to smooth domed opaque colonies (Reddy *et al.* 1994). Colonial morphology, while not necessarily a fixed property for an individual strain, may be relevant because it is thought to be related to the glycopeptidolipid component of the mycobacterial cell wall. Electron microscopy of infected macrophages shows phagocytosed organisms surrounded by a multilamellar electron-translucent zone which resembles glycopeptidolipid. It has been suggested that this zone may form a barrier which prevents intracellular killing of the organisms (Shiratsuchi *et al.* 1994). Possession of plasmids has also been correlated with virulence in the beige mouse model (Gangadharam *et al.* 1988) and plasmids have been found in strains isolated from AIDS patients with disseminated MAC disease (Crawford & Bates 1986). However, there is considerable heterogeneity in size of the plasmids identified, and the nature of the gene or genes present on plasmids relevant to virulence is unknown. Strains of *M. avium* containing the insertion sequence IS901 have been found to be more virulent in BALB/c mice than related strains lacking IS901 (Kunze *et al.* 1991), but this insertion sequence appears to be absent in isolates from AIDS patients.

1.3.5 Clinical features of disseminated *M. avium* complex infection

It is not clear why some patients suffer debilitating symptoms from the onset of disseminated MAC infection whereas others have persistent bacteraemia and appear to remain asymptomatic. Differences in virulence between strains, or in the immune response to the infecting organism by different patients may be responsible. The most commonly reported symptoms of disseminated MAC infection are low-grade fever, sweats, anorexia, weight loss and malaise. Abdominal pain and diarrhoea are also sometimes present, and less often hepatosplenomegaly and lymphadenopathy (Bermudez *et al.* 1992, Havlik *et al.* 1992, Chin 1993). These symptoms and signs are not specific to this condition and may also be found in other opportunistic infections characteristic of AIDS. The laboratory abnormalities most often identified in patients with disseminated MAC infection are anaemia and elevated serum alkaline phosphatase levels (Havlik *et al.* 1992). A model has been proposed for predicting MAC bacteraemia in patients with chronic fever and CD4 cell counts of $\leq 50/\mu\text{l}$ (Chin *et al.* 1994b).

1.3.6 Typing methods for *M. avium* complex

As with *M. tuberculosis*, a discriminative typing system which is able to distinguish between different strains of MAC would further understanding of the epidemiology of infections caused by this organism. In particular, a suitable system may be used to investigate the sources and modes of transmission of MAC, to confirm that most cases follow recent acquisition of the organism rather than reactivation of latent infection, and to gain evidence for possible outbreaks of MAC infection among susceptible individuals.

Methods based on phenotype

Serotyping has been used to investigate MAC strains isolated from human sources, including patients with AIDS, and from the environment (Tsang *et al.* 1992). In general however only a limited number of serotypes, predominantly 4, 8 and 1 have been found in AIDS patients, and this typing method is not discriminating enough to be useful. Thin layer chromatography also separates strains on the basis of their glycolipid content and has been used to investigate false positive MAC cultures arising from carry-over contamination with a BACTEC system (Bignardi *et al.* 1994). Other phenotypic methods investigated for possible use in typing are multi-locus enzyme electrophoresis (Wasem *et al.* 1991) and susceptibility to mycobacteriophages. However, these methods also suffer the drawbacks of limited discriminatory power or inability to type all strains.

Restriction fragment length polymorphism analysis

Methods based on analysis of genotype are more likely to overcome these problems. One approach is RFLP analysis using a probe to an insertion sequence of appropriate mobility and copy number, as described above for *M. tuberculosis*. A number of insertion sequences have been identified so far in the *M. avium* complex. IS900 is present in *M. paratuberculosis*, while the related IS901 and IS902 are found in *M. avium*. Possession of IS901 has been used to subdivide *M. avium* strains into type A, which lack IS901 and are the predominant type in AIDS patients, and type A/I which have multiple copies (McFadden *et al.* 1992). A/I strains have been shown to be more virulent in BALB/c mice in limited studies (Kunze *et al.* 1991). Plasmid pMB22 contains a single copy of IS900 and was identified in a genomic library constructed from a strain of *M. paratuberculosis* originally isolated from a patient with Crohn's disease (McFadden *et al.* 1987). RFLP analysis of MAC strains using the restriction endonuclease *Pvu* II and probe pMB22 suggested that only a limited number of RFLP types exist and that one particular type predominates in strains isolated from AIDS patients (Hampson *et al.* 1989, Visuvanathan *et al.* 1992). However, these results are contradicted by more recent work using probes to another insertion sequence IS1245, and by studies involving separation of large restriction fragments by pulsed-field gel electrophoresis, discussed below. These suggest that there is a high degree of polymorphism of strains isolated from different patients. It is likely that IS900 has a very low degree of mobility, which is responsible for the appearance of a conserved strain of MAC in AIDS patients, and which restricts its use as a probe in epidemiological studies.

By contrast, insertion sequence IS1245 appears promising for use in such work. This insertion sequence of 1313 base pairs belongs to the same family as IS1081 in *M. bovis* and is present in high copy number in the *M. avium* group but not in *M. intracellulare* (Guerrero *et al.* 1995). When used for RFLP analysis, human isolates contained a median of 16 copies of IS1245 and gave a diversity of patterns comparable to that obtained with pulsed-field gel electrophoresis (PFGE) (Guerrero *et al.* 1995). In some cases isolates sharing indistinguishable PFGE patterns differed in the position of some of their IS1245 copies. This may reflect superior discriminative power of IS1245 RFLP analysis compared to PFGE. Alternatively it may mean that the stability of the insertion sequence is limited, which would be a disadvantage in epidemiological work.

Two other insertion sequences evaluated in *M. avium* are IS1110, a highly mobile genetic element (Hernandez Perez *et al.* 1994), and IS1311, which shows 85% DNA homology with IS1245 (Roiz *et al.* 1995). When compared to IS900 as a probe for RFLP analysis, IS1311 gave banding patterns with all of 75 clinical isolates of *M. avium*, whereas fewer than 25% of isolates yielded RFLP patterns with IS900. Strains from patients with AIDS displayed marked polymorphism (Roiz *et al.* 1995). A rapid method for typing isolates of MAC by the polymerase chain reaction has been described recently (Picardeau & Vincent 1996). This is based on amplification of genomic sequences located between insertion sequences IS1245 and IS1311 and appears to be able to discriminate between strains to the same extent as IS1245 RFLP analysis. Although further evaluation is required, the method, which only requires a crude bacterial lysate as DNA template, is easier to perform than RFLP analysis or

PFGE and may be useful for the initial examination of large numbers of strains in epidemiological studies.

Separation of large restriction fragments by pulsed-field gel electrophoresis

An alternative approach to the determination of strain relatedness in MAC is the analysis of large restriction fragments using pulsed-field gel electrophoresis (PFGE). Chromosomal DNA analysis using conventional restriction endonucleases has not been successful because the many restriction fragments produced result in extremely complex patterns (Wards *et al.* 1987). These can be simplified by employing restriction endonucleases which cut the chromosomal DNA infrequently, usually at fewer than 10 recognition sites per 10^6 base pairs, giving rise to a small number of large restriction fragments. Mycobacteria are known to have a high proportion (62 - 70 mol%) of guanine plus cytosine in their genomic DNA, so that restriction enzymes which have 6-base recognition sites rich in adenine and thymine are likely to cut the genomic DNA suitably infrequently. To prevent cleavage of chromosomal DNA and large restriction fragments by shearing stresses, bacterial cells are embedded in agarose before enzymatic treatment to lyse the cell walls and allow penetration of the restriction endonuclease. Large restriction fragments up to two megabases in size are then separated by PFGE, in which a homogeneous electric field through the gel alternates between two orientations 120° apart throughout the electrophoresis run (Chu *et al.* 1986). Conventional electrophoresis is unable to separate fragments of this size. Migration of fragments in the gel depends on the potential difference across the gel, the agarose concentration and buffer composition, the switch time between changes in angle of the electric field, and the size of the DNA fragments. Large fragments move

proportionately more slowly than small ones partly because it takes longer for them to reorientate themselves with respect to the changing electric field. The switch times selected for each electrophoretic run may be chosen for optimum resolution of DNA fragments in a particular range of molecular sizes. This technique has been used for a wide range of Gram positive and Gram negative bacteria and for mycobacteria including *M. tuberculosis* (Zhang *et al.* 1992, Olson *et al.* 1994), *M. bovis* (Feizabadi 1996), *M. bovis BCG* (Zhang *et al.* 1995), *M. fortuitum* (Hector *et al.* 1992) and *M. haemophilum* (Yakrus *et al.* 1994). Compared to RFLP analysis PFGE has the advantage that a labelled probe, such as to an insertion sequence, is not required, and therefore the method may in theory be applied to any species without detailed knowledge of its genomic DNA.

PFGE and field inversion gel electrophoresis, a precursor of PFGE, have been used to study the taxonomic relationships of the *M. avium* complex. Wood pigeon mycobacteria form a homogeneous group that is distinct from *M. paratuberculosis*; *M. avium* and *M. intracellulare* strains may be distinguished (Levy-Frebault *et al.* 1989, Coffin *et al.* 1992). Mazurek *et al.* (1993) studied large restriction fragment (LRF) patterns of 35 randomly selected clinical isolates of MAC. The restriction endonucleases *Asn* I and *Xba* I gave clear patterns more consistently than other enzymes tested. The patterns for unrelated strains were highly polymorphic and each strain had a unique pattern which appeared to be stable. These properties indicate the suitability of the method for epidemiological studies of MAC infection. It was also shown that multiple isolates recovered from the same patient from different sites and/or over an extended period gave indistinguishable LRF patterns. These

observations were confirmed and extended by Arbeit *et al.* (1993), who isolated *M. avium* from prospective cultures on 14 HIV-infected patients. Although serotyping and ribotyping were of limited use in discriminating between isolates from different patients, LRF patterns produced by *Ase* I (analogous to *Asn* I) and resolved with PFGE showed that each patient was infected with a unique strain. Such genetic diversity supports the hypothesis that MAC infection in AIDS patients is usually acquired from a variety of environmental sources. Furthermore, of 13 patients with MAC bacteraemia two patients had concurrent infection with two distinct strains. Subsequent work showed that 4 out of 12 patients from whom sequential cultures were collected within a two week period had two distinct MAC strains, which was suggested to reflect ongoing polyclonal infection (Slutsky *et al.* 1994). This observation is relevant when multiple strains in a single patient differ in antimicrobial susceptibility, as demonstrated for four out of five patients with polyclonal *M. avium* infection (von Reyn *et al.* 1995). Treatment directed at one strain could result in selection of the other resistant strain under these circumstances.

PFGE was used in a prospective study which identified hospital hot water supplies as a source of *M. avium* infection in AIDS patients (von Reyn *et al.* 1994). 81% of 36 patients examined were infected with their own unique strain of *M. avium*, but three clusters of two or three patients with indistinguishable strains were also found. For two of the clusters the same strain was also isolated repeatedly from the recirculating hot water system of the hospitals attended by the patients, who had no other common exposures.

1.4 SCOPE OF THE STUDY

M. tuberculosis

The epidemiology of mycobacterial disease in patients infected with HIV has been well documented in some countries, especially the United States. However, much less was known about the United Kingdom situation at the commencement of this study (1993). With regard to *M. tuberculosis*, there are several questions to be addressed. To what extent has HIV co-infection contributed to the increase in notifications of tuberculosis in Britain in recent years? Is there evidence for outbreaks of tuberculosis among patients in HIV units as observed elsewhere, and are these patients more likely to be infected with drug resistant strains? Studies abroad have suggested that the proportion of cases of active tuberculosis due to recently acquired infection rather than reactivation of old disease is higher than expected. Does the same apply to HIV patients in the United Kingdom? While conventional epidemiological methods may help to answer some of these points, the application of molecular biological techniques, especially RFLP analysis to a suitable sample of strains is needed to resolve these issues.

M. avium complex

The epidemiology and pathogenesis of disseminated MAC infection are less clearly established than for tuberculosis. In particular, the degree of heterogeneity among strains causing disseminated infection in HIV patients in the United Kingdom is unknown. The portal of entry of the organism is often unclear, but might be inferred in some cases if strains causing disseminated infection were found to be similar to

those isolated from superficial sites such as the gastrointestinal or respiratory tracts. It is unknown whether patients with persistent MAC infection over a period of time have the same strain throughout or a sequence of infections with different strains. Most cases are assumed to arise from sporadic infection in a susceptible individual from an environmental source, but the occurrence of common source outbreaks in HIV units has not been sought in Britain, although there is evidence that small outbreaks have occasionally happened in the United States. Again, these questions can be investigated further by applying a discriminative typing method to an appropriate sample of stains.

Location of the study

The Communicable Disease Surveillance Centre (CDSC) of the Public Health Laboratory Service (PHLS) has a voluntary confidential reporting system for cases of HIV infection and AIDS. So far, more cases have been reported from the Thames regions than from any other part of the United Kingdom: 16253 persons infected with HIV out of a total of 25276 up to the end of September 1995 (CDSC 1995a), and 8281 AIDS cases out of 11872 between January 1982 and December 1995 (CDSC 1996b). Data from unlinked anonymous HIV testing among patients attending genitourinary medicine clinics and women giving birth also confirm that the prevalence of HIV-1 infection is much higher (range 4.0 to 19.1 times) in London and the South East than elsewhere in England and Wales (CDSC 1996c). Most of the microbiology departments serving hospitals in the South East (roughly, the area east of Oxford and South of Cambridge and including Greater London), including all but one of the laboratories associated with the HIV units in London, send mycobacteria

isolated from clinical specimens to the Mycobacterium Reference Unit at Dulwich Public Health Laboratory for identification and susceptibility testing (Figure 1.4.1). Isolates from persons identified as HIV positive by the referring laboratory have been stored since the early 1980s. This collection of stored strains, which is likely to be the most extensive and representative collection of mycobacteria isolated from HIV patients in South East England, was used in this study.



Figure 1.4.1

South East England, showing the location of laboratories which submitted isolates of mycobacteria to Dulwich Public Health Laboratory for identification and susceptibility testing in 1994. In addition to those shown, isolates were also received from 31 laboratories in Greater London as well as from Croydon, Kingston and Sidcup.

2. METHODS

2.1 MYCOBACTERIUM TUBERCULOSIS

This study used cultures of *M. tuberculosis* isolated from known HIV seropositive patients and referred by laboratories in South East England to the Regional Tuberculosis Centre at Dulwich Public Health Laboratory between January 1991 and June 1994. As they were received, the identification of the isolates was confirmed and their susceptibility to first line antituberculous drugs determined, as part of the routine work of the reference laboratory. The isolates were then stored on 2 ml Lowenstein-Jensen slopes at room temperature until required. All work on live cultures of *M. tuberculosis* was performed in a Class 1 safety cabinet within Category 3 accommodation.

2.1.1 Identification of *M. tuberculosis*

For each culture, a film was prepared and stained by the Ziehl-Neelsen method to confirm the presence of acid-fast bacilli. A smooth suspension of cells was then obtained by stirring a 10 μ l loopful of culture into 1 ml phosphate-buffered saline with large glass beads on a magnetic stirrer for several minutes. This suspension was used to inoculate three Lowenstein-Jensen slopes: one was incubated at 25°C, one at 37°C and exposed to light, the third contained 500 mg/l p-nitro benzoic acid (PNBA) and was also incubated at 37°C. In these screening tests members of the *M. tuberculosis* complex do not grow at 25°C, do not produce pigment, are inhibited by PNBA, and visible growth is not apparent within three days. The subspecies of the

M. tuberculosis complex (*M. t. tuberculosis*, *africanum*, *bovis*, *BCG*) may be distinguished by a further battery of tests (Collins *et al.* 1985). These include preference for aerobic or microaerophilic growth, preference for growth on Lowenstein-Jensen slopes containing pyruvate or glycerol, susceptibility to 5 mg/l thiophen-2-carboxylic acid hydrazide (TCH), 25 mg/l cycloserine, and 66 mg/l pyrazinamide (PZA), and ability to reduce nitrate to nitrite (Table 2.1). Only isolates of *M. tuberculosis* were investigated in this study.

Table 2.1.1 Identification of *M. tuberculosis* subspecies

Subspecies	Oxygen preference	Pyruvate: Glycerol	TCH	Cyclo-serine	PZA	Nitrate reduction
Classical	A	P = G	R	S	S	+
South Indian	A	P = G	S	S	S	+
African I	M	P > G	S	S	S	-
African II	M	P > G	S	S	S	+
Bovis	M	P > G	S	S	R	-
BCG	A	G > P	S	R	R	v

A aerobic, M microaerophilic, R most strains resistant, S most strains susceptible, + positive reaction, - negative reaction, v variable

2.1.2 Susceptibility testing

The susceptibility of isolates to streptomycin, isoniazid, ethambutol and rifampicin was determined by the resistance ratio method (Collins *et al.* 1985).

A series of Lowenstein-Jensen slopes containing doubling dilutions of each drug to be tested was prepared. The final drug concentrations used were as follows:

streptomycin 40 to 2.5 mg/l, isoniazid 0.2 to 0.125 mg/l, ethambutol 3.2 to 0.2 mg/l, and rifampicin 50 to 3.125 mg/l. 5 μ l of the bacterial suspension prepared above was inoculated onto each tube and onto one drug-free slope. Control strains of *M. tuberculosis* of known susceptibility were also tested. The slopes were incubated at 37°C and read after 10 days, or a further 7 days if growth on the drug-free slope was initially poor. The slope containing the lowest concentration of drug to yield fewer than 20 colonies of the test strain after this time was taken as the end-point. Strains were regarded as sensitive to the drug if the ratio of the minimum inhibitory concentration (MIC) of the test strain to the MIC of the control strain was 2 or less, and resistant if the ratio of the MICs was 4 or more.

Susceptibility to pyrazinamide was tested in a mixed solid/semi-solid medium at acid pH. Tubes containing 1 ml of Lowenstein-Jensen medium, pH 5.2 with 66 mg/l pyrazinamide were prepared, and layered with 2 ml of semi-solid Kirchner medium containing 8% oleic acid-albumin-dextrose-catalase (OADC) enrichment (Becton-Dickinson), 3 g/l sodium pyruvate, 1 g/l agar and 66 mg/l pyrazinamide, pH 5.2. Similar tubes without pyrazinamide were also made up. One pair of tubes was inoculated with 20 μ l of bacterial suspension and one pair with 20 μ l of a 1 in 10

dilution of the suspension. The test strain was reported as susceptible to pyrazinamide if growth occurred in the control tube but not in the drug-containing tube with either the original or the diluted suspension.

2.1.3 Subculture of stored strains

From June 1993 onwards each stored strain was subcultured onto a 5 ml Lowenstein-Jensen slope as well as in 2 ml Middlebrook 7H9 broth (Difco) containing 10% OADC supplement. If no growth was visible on the slope after 6 weeks incubation at 37°C, the broth cultures were subcultured to further Lowenstein-Jensen slopes. Strains were considered to be non-viable if no growth was apparent after 12 weeks incubation. Slopes yielding sufficient growth were harvested using a cotton swab moistened in sterile distilled water to pick up as many colonies as possible. These were dispersed by agitating the swab in 0.6 ml Tris EDTA (TE) buffer (10 mM Tris, 1 mM EDTA, pH 8.0) in a labelled microcentrifuge tube.

2.1.4 Restriction fragment length polymorphism analysis

The method used for RFLP analysis of *M. tuberculosis* is based on that of van Soolingen *et al.* (1993b) with modifications, and conforms with the recommendations for a standardised methodology for this procedure proposed by an international group (van Embden *et al.* 1993).

Preparation of chromosomal DNA

The harvested cells were killed by heating the microcentrifuge tubes in a water bath at 80°C for at least 20 minutes. When cool, the cell walls were lysed by incubating with lysozyme (Boehringer Mannheim, 1 mg/ml final concentration) for 90 minutes at 37°C followed by proteinase K (Boehringer Mannheim, 0.2 mg/ml) and 1% sodium dodecyl sulphate (Sigma) for 30 minutes at 65°C. Cell wall debris, denatured protein and polysaccharide were then removed by complexing with cetyl-dimethyl ethyl ammonium bromide (Sigma, 10 mg/ml) in the presence of sodium chloride, followed by extraction with chloroform : isoamyl alcohol (24:1). After centrifugation, the upper layer of aqueous supernatant was pipetted to a fresh tube and the DNA precipitated with an equal volume of isopropanol at -20°C. The precipitate was washed in cold 70% ethanol then redissolved in 20 μ l TE buffer and stored at 4°C.

Restriction digestion of chromosomal DNA

10 μ l of each DNA preparation was digested with 10 units of restriction endonuclease *Pvu* II (Gibco), using the restriction buffer provided with the enzyme. The incubation was carried out for 4 hours at 37°C in a total volume of 20 μ l, then the reaction was stopped by adding 5 μ l of loading buffer (30% glycerol, 0.15% bromophenol blue, EDTA) containing internal size markers.

To assess the concentration of DNA in the samples and to check digestion 5 μ l of each preparation was applied to the slots of a 0.8% agarose gel in TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA) containing 0.5 mg/l ethidium bromide. 1 μ g of lambda phage DNA digested with *Hind* III (Gibco BRL) was also applied to

the gel as a molecular weight marker. Electrophoresis was carried out at 80V for one hour before taking a Polaroid photograph of the gel on an ultraviolet transilluminator. From this photograph the volume of each preparation containing 1 μ g DNA for Southern blotting was estimated.

Electrophoresis and Southern transfer of the DNA restriction fragments

Electrophoresis of the fragments was carried out in a 20 x 20 cm 0.8% agarose (DNA grade, Biorad) gel containing 0.5 mg/l ethidium bromide. The gel was submerged in TAE buffer and loaded with up to 24 samples, each containing approximately 1 μ g DNA, plus molecular weight markers (lambda *Hind* III fragments end-labelled with digoxigenin-dUTP (Boehringer Mannheim) using Klenow DNA polymerase) at each end. The electrophoresis was run at 25 volts for approximately 16 hours, until the 2.0 kilobase fragment of the molecular weight marker had migrated 7 cm from the wells, when viewed on an ultraviolet transilluminator. The gel was exposed to ultraviolet light for 5 minutes then immersed in 0.25 M hydrochloric acid for 20 minutes. These steps nicked and depurinated the DNA fragments, facilitating their subsequent transfer by blotting. After rinsing in distilled water the gel was incubated twice for 15 minutes in denaturing solution (0.5 M sodium hydroxide, 1.5 M sodium chloride) to denature double stranded DNA, then for 30 minutes in neutralising solution (1.5 M sodium chloride, 1.0 M Tris, pH 7.4) before blotting. For this, the gel was placed on a sheet of Hybond-N nylon membrane (Amersham International) moistened with 2 x SSC (0.3 M sodium chloride, 0.03 M sodium citrate, pH 7.0) on a Hybaid vacuum blotter. The gel was covered with 2 x SSC and a vacuum applied for 60 minutes to transfer DNA fragments to the membrane. The DNA was then crosslinked to the

membrane by exposure to 120 000 $\mu\text{J}/\text{cm}^2$ in an ultraviolet Stratalinker 1800 (Stratagene) before hybridisation.

Preparation of IS6110 probe by PCR

A probe to IS6110, labelled with digoxigenin, was prepared in a polymerase chain reaction. The reaction mix contained 5 μl target DNA (pRP 2001, a plasmid containing IS6110), 5 μl each of primers ISTB5 and ISTB6 (a gift of Dr P Godfrey-Faussett), deoxy-nucleoside triphosphates (2.5 μl of 2 mM dATP, dCTP and dGTP plus 3.3 μl of 1 mM dTTP), 1.7 μl digoxigenin-11-dUTP, 2 μl dimethylsulphoxide, 5 μl 10x Cetus buffer (500 mM potassium chloride, 15 mM magnesium chloride, 100 mM Tris, pH 8.3) and 0.5 μl *Taq* DNA polymerase (5 U/ μl , Boehringer-Mannheim), made up to 50 μl with sterile distilled water. The reaction mix was overlaid with mineral oil then subjected to the following conditions in a thermal cycler: 3 minutes at 90°C, followed by 30 denaturing/annealing/synthesising cycles of 90°C for 40 seconds/50°C for 40 seconds/72°C for 2 minutes, and finally 10 minutes at 72°C.

At the end of the polymerase chain reaction, the entire reaction mix was loaded onto a gel of 0.8% low melting point agarose (Biorad) containing ethidium bromide and electrophoresis was carried out at 80 volts for one hour. The gel was then viewed on an ultraviolet transilluminator and the band corresponding to the labelled product was cut from the gel with a sterile scalpel blade, and weighed in a microcentrifuge tube. Sufficient sterile distilled water was added to dilute the agarose to 0.2%, which was then melted by heating at 65°C. The preparation was stored in 100 μl aliquots at 4°C.

To assess the activity of the labelled probe prepared in this way, 10 μ l of pRP2001 DNA (containing IS6110) was crosslinked to four pieces of Hybond-N membrane which were sealed into small plastic envelopes each containing 5 ml pre-hybridisation solution. After incubating for one hour at 65°C different quantities of probe (2, 10, 25 and 50 μ l) which had been boiled and cooled on ice were added to each envelope. Hybridisation was carried out at 65°C overnight. The membranes were then washed and digoxigenin detected in a chemiluminescent reaction, as described below. The optimum concentration of probe was 2 μ l per 5 ml hybridisation solution.

Hybridisation

The crosslinked membrane was moistened with 2 x SSC and inserted into a clean glass hybridisation bottle. 25 ml prehybridisation solution (5 x SSC, 0.1% N-lauroyl sarcosine (Sigma), 0.5% sodium dodecyl sulphate, 3% dried skimmed milk (Safeway)) warmed to 65°C and containing 0.5 ml herring sperm DNA solution (5 mg/ml, Sigma) which had been boiled then cooled on ice, was added to the bottle. Prehybridisation continued for one hour at 65°C in a rotating hybridisation oven (Hybaid), then 10 μ l of digoxigenin-labelled probe to IS6110, made single stranded by boiling for 5 minutes then chilling on ice, was added to the bottle. After hybridisation at 65°C overnight, the hybridisation mix containing probe was removed and the membranes washed twice in a solution of 2 x SSC, 0.1% sodium dodecyl sulphate, 0.02% N-lauroyl sarcosine and once in TBS (0.1 M Tris, 0.15 M sodium chloride, pH 7.5).

Chemiluminescent detection of IS6110 probe

Non-specific protein binding sites on the membrane were blocked by incubating in 25 ml of 3% milk solution (in TBS) for one hour at 37°C. Antibody to digoxigenin conjugated to alkaline phosphatase (Boehringer-Mannheim, 2.5 µl in 25 ml of freshly prepared 3% milk solution containing 0.5% Tween 20) was then added and incubated at 37°C for a further 30 minutes. After washing six times in TBS containing 0.5% Tween 20 the membrane was equilibrated in alkaline phosphatase reaction buffer (0.1 M sodium chloride, 0.01 M magnesium chloride, 0.1 M Tris, pH 9.5) for five minutes at room temperature. Lumiphos-540 (Boehringer-Mannheim), a substrate of alkaline phosphatase which gives out light when converted to product by the enzyme, was applied to the surface of the membrane which was then sealed into a clear plastic bag. After preincubation at 37°C for 30 minutes to allow the enzyme reaction rate to increase, the bag was sealed into an exposure cassette with an X-ray film (Hyperfilm MP, Amersham International) and reincubated at 37°C. After an exposure of approximately 90 minutes, the film was removed and developed (Kodak GBX developer and fixer solutions). A shorter or longer exposure was sometimes required depending on the intensity of bands on the resulting autophotograph.

Preparation and detection of internal size markers

In order to facilitate comparison between different gels internal molecular weight markers were included in the tracks of every DNA sample, as recommended by van Soolingen *et al.* (1993). These were prepared from a supercoiled DNA ladder (Gibco BRL) digested with *Pvu* II added to *PhiX* 174-*Hae* III DNA (Gibco BRL), to give a ladder of about 15 resolvable bands between 16.2 and 0.6 kilobases in size.

After the first hybridisation, the membrane was returned to a roller bottle and the probe to *IS6110* removed by incubating twice with 0.4 M sodium hydroxide, 0.1% sodium dodecyl sulphate at 45°C for 30 minutes, then rinsing twice in 2 x SSC. Prehybridisation was carried out as above, then a second hybridisation with digoxigenin-labelled probes to the internal size markers continued overnight at 65°C. These probes (10 µl of each in 25 ml prehybridisation solution) were pUC9.2 for the supercoiled DNA fragments and PhiX 174-*Hae* III fragments, both labelled with digoxigenin by random priming using T7 DNA polymerase (Gibco BRL), random nona-nucleotides, and a mixture of unlabelled deoxynucleoside triphosphates plus digoxigenin-11-dUTP (Boehringer-Mannheim). Finally, the membrane was washed and probes from the second hybridisation detected using anti-digoxigenin antibody conjugated to alkaline phosphatase and Lumiphos-540 chemiluminescent substrate as described above.

2.1.5 Computer analysis of *IS6110* restriction fragment length polymorphism patterns

The autophotographs showing the *IS6110* RFLP patterns of the isolates were scanned using a Hewlett Packard Scanjet 3C scanner and the data loaded into a Compu-Add CP90P computer set up with Gelcompar for Windows software (Applied Maths BVBA). After converting each gelscan into Gelcompar format, the patterns were normalised by aligning the reference positions (lambda *Hind* III markers) at both ends of each gelscan with a pre-selected standard reference track. This allowed comparison

between gelstrips on different gelscans. Although it was originally intended to use the internal molecular weight markers for normalisation of the gels, use of the lambda *Hind* III markers on the same scan was generally satisfactory and considerably simpler than normalisation with two scans for each gel. Each gelstrip was then inspected to ensure that the peaks selected by densitometric scanning corresponded with the position of the bands on the original autophotograph. Two lists of isolates to be compared, one of isolates from HIV seropositive patients and one of isolates from controls, were drawn up. The degree of similarity between tracks in each list based on the position of assigned bands was calculated using the coefficient of Jaccard, and the tracks were clustered in the form of dendrograms by the unweighted pair group method using arithmetic averages.

2.2 MYCOBACTERIUM AVIUM COMPLEX

2.2.1 Selection, identification and storage of isolates

HIV seropositive patients with disseminated *M. avium* complex (MAC) infection were identified from the records of isolates referred to the Regional Tuberculosis Centre at Dulwich Public Health Laboratory between December 1991 and June 1994. Patients with more than one available isolate, including at least one grown from a blood culture or bone marrow specimen, were selected.

Isolates were identified as MAC at the time of referral to the laboratory by the following characteristics: small coccoidal acid fast bacilli on Ziehl-Neelsen stain, slow growth (> 3 days) at 25°C, 37°C and variably at 44°C, failure to hydrolyse Tween 80 and to reduce nitrate. They were then stored on small Lowenstein Jensen slopes at room temperature.

2.2.2 Separation of large restriction fragments by pulsed-field gel electrophoresis

Subculture of isolates

Each isolate was subcultured by inoculating 10 ml of Middlebrook 7H9 broth (Difco) containing 10% OADC supplement (Becton-Dickinson) and 0.05% Tween 80 and incubating at 37°C until the late exponential phase of growth, 5 to 10 days. The cultures were centrifuged at 3000 rpm for 10 minutes, then the pellet was resuspended

in 200 μ l of TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) and heated at 80°C for 30 minutes.

Preparation of agarose plugs and liberation of intact mycobacterial DNA

This was a modification of the method of Mazurek *et al.* (1993). 50 μ l of cell suspension was mixed with an equal volume of 2% low melting point agarose (Biorad) and transferred to a plug mold. When the plug had set it was transferred to a microcentrifuge tube containing 500 μ l of lysozyme (Boehringer-Mannheim) 1 mg/ml in TE buffer, and incubated at 37°C for 2 hours. The solution was then aspirated and replaced with 500 μ l of TE buffer containing 0.2 mg/ml proteinase K (Boehringer-Mannheim) and 1% sodium dodecyl sulphate (Sigma) and incubated at 50°C overnight. The plugs were then washed four times in TE buffer (4 x 1 hour) at room temperature. 1 mM phenylmethyl sulphonyl fluoride (Sigma), freshly prepared from stock solution was added to the second wash to neutralise remaining proteinase activity. Plugs were stored in TE buffer at 4°C until required.

Restriction endonuclease digestion

Half of each plug was incubated in 500 μ l of restriction enzyme buffer (NEB 3 for *Ase* I: 100 mM sodium chloride, 50 mM Tris-HCl, 10 mM magnesium chloride, 1 mM dithiothreitol, pH 7.9) in a microcentrifuge tube for one hour at room temperature. The buffer was then aspirated and replaced with 100 μ l of fresh restriction enzyme buffer containing 10 units (1 μ l) *Ase* I (New England Biolabs). Digestion was carried out at 37°C overnight. Some strains were also digested with 20 units *Xba* I (New England Biolabs) in NEB 2 buffer (50 mM sodium chloride, 10 mM

Tris-HCl, 10 mM magnesium chloride, 1 mM dithiothreitol, pH 7.9) under the same conditions.

Pulsed-field gel electrophoresis

Each plug containing restriction digested DNA was equilibrated in 500 μ l electrophoresis buffer (0.5 x TBE: 45 mM Tris, 45 mM borate, 1.0 mM EDTA, pH 8.3) for 30 minutes at room temperature, then transferred to a tooth on a 15 tooth comb held in a gel casting stand. Agarose plugs containing concatamers of lambda phage DNA (size range 48.5 to 1018 kilobases, Biorad) were placed on teeth at both ends of the comb as molecular weight markers. 100 ml of pulsed-field certified agarose (Biorad, 1 g/100ml in 0.5 x TBE buffer), melted and cooled to 50°C were poured into the casting stand around the comb and allowed to set. After removing the comb from the gel the remaining wells were filled with agarose and the gel was transferred to the electrophoresis tank of a Biorad CHEF-DR II system (Clamped Homogeneous Electric Fields with Dynamic Regulation) containing two litres of 0.5 x TBE buffer. PFGE was carried out for 21 hours at 6.0 volts/cm. Switch time increased linearly from 20 seconds at the start of the run to 60 seconds at the end for plugs containing DNA digested with *Ase* I, and from 10 to 50 seconds for plugs treated with *Xba* I. The temperature was maintained at 15°C throughout by recirculating buffer through a chiller. At the end of electrophoresis the gel was stained in 0.5 μ g/ml ethidium bromide solution for 20 minutes then destained in distilled water for 2 hours. DNA fragments were visualised by placing the gel on an ultraviolet transilluminator and recorded on Polaroid photograph.

3. RESULTS

3.1 *MYCOBACTERIUM TUBERCULOSIS*

Viability of stored strains

Between January 1988 and June 1994 inclusive 269 isolates of mycobacteria from HIV seropositive patients were referred to the PHLS Regional Tuberculosis Centre at Dulwich and were confirmed as *M. tuberculosis*. When these stored isolates were subcultured (starting in June 1993) their viability was found to be dependent on the age of the cultures (Table 3.1.1). Overall, 89% of isolates less than a year old at the time of subculture were viable, whereas 42% of cultures between one and two years old, and only 11% of cultures more than two years old were still alive.

Table 3.1.1 Viability of stored isolates of *M. tuberculosis* referred from HIV seropositive patients.

Year	Number of cultures received	Number (%) of cultures viable on subculture ^a
1988	13	0 (0)
1989	35	4 (11.4)
1990	28	4 (14.3)
1991	39	5 (12.8)
1992	73	51 (69.9)
1993	42	37 (88.1)
1994 ^b	39	35 (89.7)
Total	269	136

^a commenced June 1993

^b January to June

Numbers of cases and controls

128 of the viable isolates, obtained from 96 HIV patients, were studied further.

Between one and three isolates were referred from each patient. The laboratories from which these isolates were referred, all in London or South East England, are listed in Table 3.1.2.

For comparison, 90 further isolates from 84 patients not identified as HIV seropositive by the referring laboratory were selected as controls. These were received between February and September 1993 from the same laboratories that had sent isolates from HIV patients (Table 3.1.2).

69% of the HIV positive cases were male compared to 59% of the controls. The age of the cases at the time that tuberculosis was diagnosed was known for 81 out of 96 HIV patients and for 64 of the 84 controls. The median age at diagnosis was 35 years for HIV cases (standard error 8.8 years) and 39 years (standard error 15.7 years) for the controls.

Table 3.1.2 Laboratories from which the *M. tuberculosis* isolates used in this study were referred.

Referring laboratory	Number of HIV positive cases	Number of control cases
Chelsea and Westminster Hospital*	20	17
University College Hospital	18	31
St. George's Hospital	7	2
Charing Cross Hospital	5	0
King's College Hospital	5	5
Royal London Hospital	5	4
St. Andrew's Hospital	5	2
North Middlesex Hospital	4	2
Royal Free Hospital	4	7
Ealing Hospital	3	2
Whipps Cross Hospital	3	1
Central Middlesex Hospital	2	3
Hammersmith Hospital	2	2
Institute of Neurology	2	0
Northwick Park Hospital, Harrow	2	3
Greenwich District Hospital	1	0
Kingston Hospital	1	0
Maidstone Hospital	1	0
Mount Vernon Hospital	1	0
Royal Sussex County Hospital, Brighton	1	0
St. Bartholomew's Hospital	1	2
Watford General Hospital	1	0
West Middlesex Hospital	1	0
Whittington Hospital	1	0
Margate Hospital	0	1
Total	96	84

* including Westminster Hospital prior to 1993

The likely ethnic origins of HIV positive cases and controls were estimated by inspection of surnames, as done by Yates *et al.* (1993) (Table 3.1.3):

Table 3.1.3 Presumed ethnic origin of HIV positive cases and controls.

Presumed ethnic origin	Number (%) of HIV seropositive cases	Number (%) of controls
European	55 (57.3)	51 (60.7)
African	33 (34.4)	18 (21.4)
Indian subcontinent	3 (3.1)	15 (17.9)
Other*	5 (5.2)	0
Total	96	84

*includes Oriental and unknown names (for example, STD clinic numbers)

Sites of infection

The sites of infection from which *M. tuberculosis* was isolated in the two groups are shown in Table 3.1.4. Pulmonary infection was the most frequent site in both groups, although the proportion of cases with pulmonary infection was higher in the control group. Isolation from blood cultures or bone marrow occurred in 16/96 (17%) HIV patients but was not found among the control group.

Table 3.1.4 Clinical sites from which *M. tuberculosis* was isolated in HIV positive patients and controls.

Site	Number (%) of HIV patients	Number (%) of controls
Pulmonary:		
sputum	35 (36)	47 (57)
bronchoalveolar lavage fluid	14 (14)	7 (8)
pleural fluid/biopsy	5 (5)	8 (10)
Lymph node, neck abscess	22 (23)	10 (12)
Blood culture, bone marrow	16 (16)	0
Urine	11 (11)	0
Stool	7 (7)	1 (1)
Gastric washing	1 (1)	0
CSF, brain abscess	2 (2)	0
Peritoneal fluid/biopsy	2 (2)	4 (5)
Bone, spine	0	4 (5)
Other (tissue biopsy)	5 (5)	6 (7)

Some patients had positive cultures from more than one site

Drug susceptibility

The susceptibility of the isolates to 'first line' antimycobacterial drugs (rifampicin, isoniazid, ethambutol, pyrazinamide and streptomycin) is shown in Table 3.1.5. The majority of isolates from both groups were susceptible to all drugs tested. 8/96 (8.3%) HIV patients and 1/84 (1.1%) controls had isolates resistant to two or more antituberculous drugs, but only two cases, both HIV positive patients, had isolates resistant to both rifampicin and isoniazid.

Table 3.1.5 Susceptibility of *M. tuberculosis* isolated from HIV patients and controls to first line antimycobacterial drugs

Susceptibility	Number of HIV patients	Number of controls
Fully susceptible (to rifampicin, isoniazid, pyrazinamide, ethambutol and streptomycin)	77	77
Resistant to one drug: isoniazid streptomycin pyrazinamide	7 3 2	3 3 0
Resistant to two drugs: isoniazid and streptomycin isoniazid and rifampicin	4 2	1 0
Resistant to three drugs: isoniazid, streptomycin and ethambutol	2	0
Total	97*	84

*includes one patient with two isolates of different susceptibilities

RFLP analysis

RFLP patterns were obtained for all 218 isolates that yielded sufficient growth.

Examples of autophotographs are shown in Plates 1 and 2. Each isolate gave at least one band when hybridised with the digoxigenin-labelled probe to IS6110, indicating that all isolates contained at least one copy of this insertion sequence. The majority of isolates had between 8 and 13 copies (Figure 3.1.1).

HIV seropositive patients

Two or three viable isolates were received in each case from 25 of the patients in this group. In most of these, isolates were from different clinical sites, forwarded to the reference laboratory at the same time. In each case, visual inspection of the RFLP patterns showed that the different isolates were indistinguishable in terms of the number, position and intensity of the bands. 23 of the 25 cases had similarity coefficients of 100% for the different isolates when analysed by Gelcompar. In the other two cases, differences in gel loading or electrophoretic migration between gels may have accounted for failure of the system to recognise similar patterns as the same.

Two isolates with different susceptibility patterns were received from one HIV positive patient. The second isolate, cultured from a sputum specimen and resistant to pyrazinamide, was referred eight months after the first one, which had been isolated from a lymph node biopsy and was fully sensitive to antimycobacterial drugs. Both

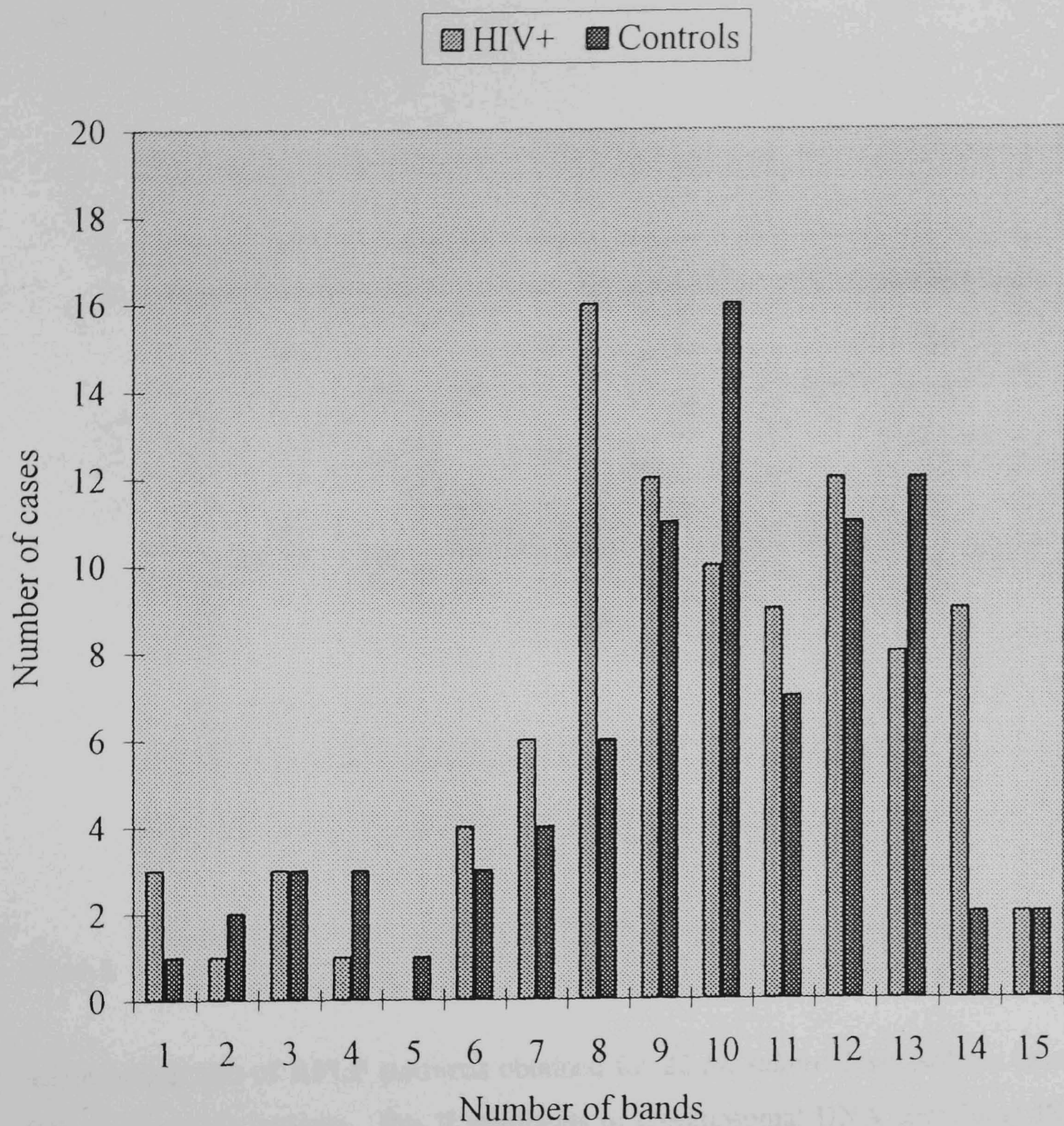


Figure 3.1.1

Distribution of bands (IS6110 copies) among *M. tuberculosis* isolates from 96 HIV seropositive and 84 control patients with tuberculosis.

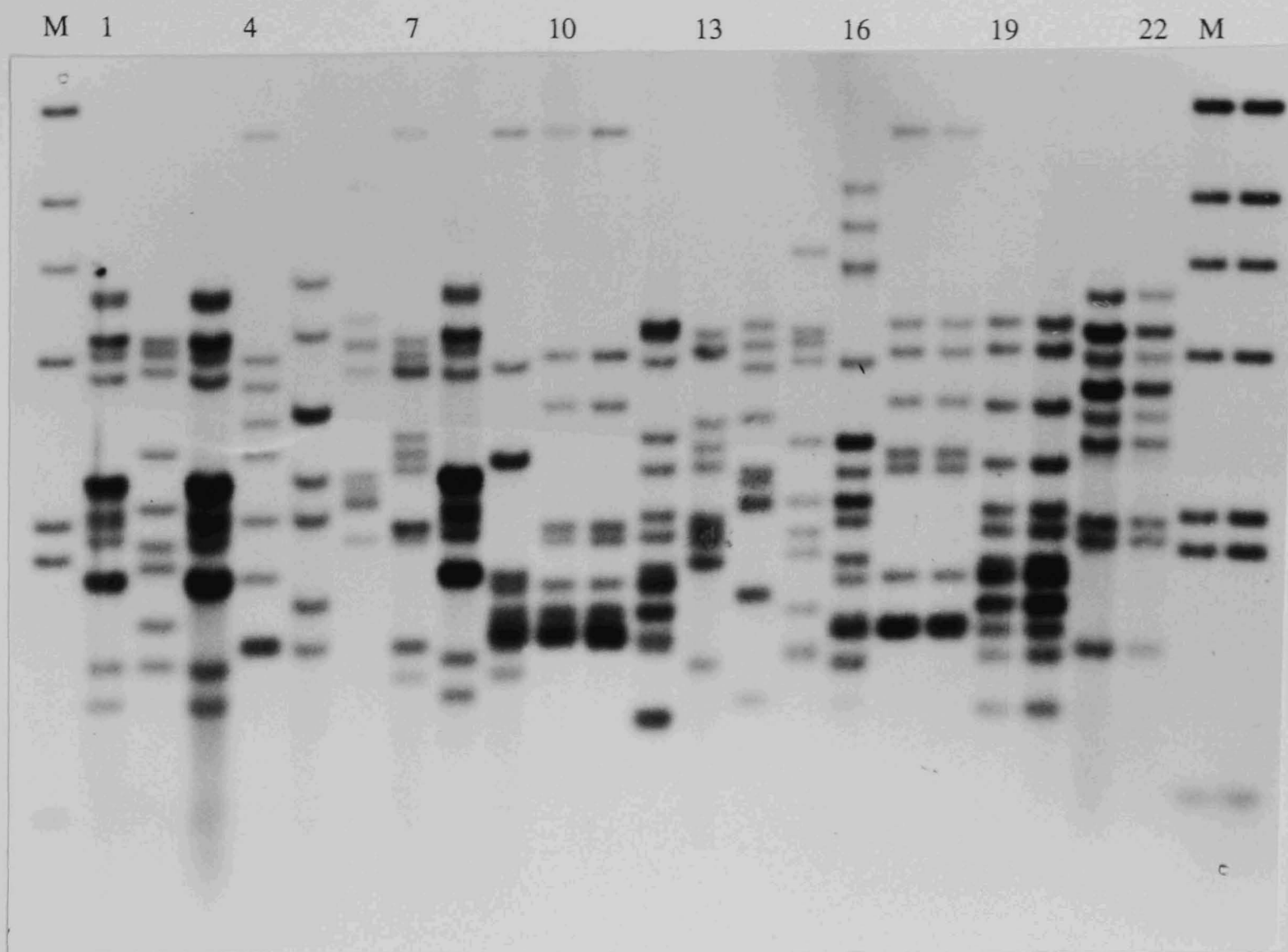


Plate 1

Autophotograph of RFLP patterns obtained for 22 *M. tuberculosis* isolates from 18 HIV seropositive patients. *Pvu* II fragments of chromosomal DNA containing IS6110 were detected with a digoxigenin-labelled probe to this insertion sequence.

The pairs of isolates in lanes 3/8, 17/18, 19/20, 21/22 were from patients with more than one positive culture (at different body sites) in each case, and appear indistinguishable. By contrast, the indistinguishable isolates in lanes 1 and 3/8 and in lanes 10 and 11 were from different patients, representing clusters of cases (patients B,A and H,I in Table 3.1.6).

M: lambda *Hind* III molecular weight marker (23.1, 9.4, 6.6, 4.4, 2.3, 2.0, 0.6 kilobases).

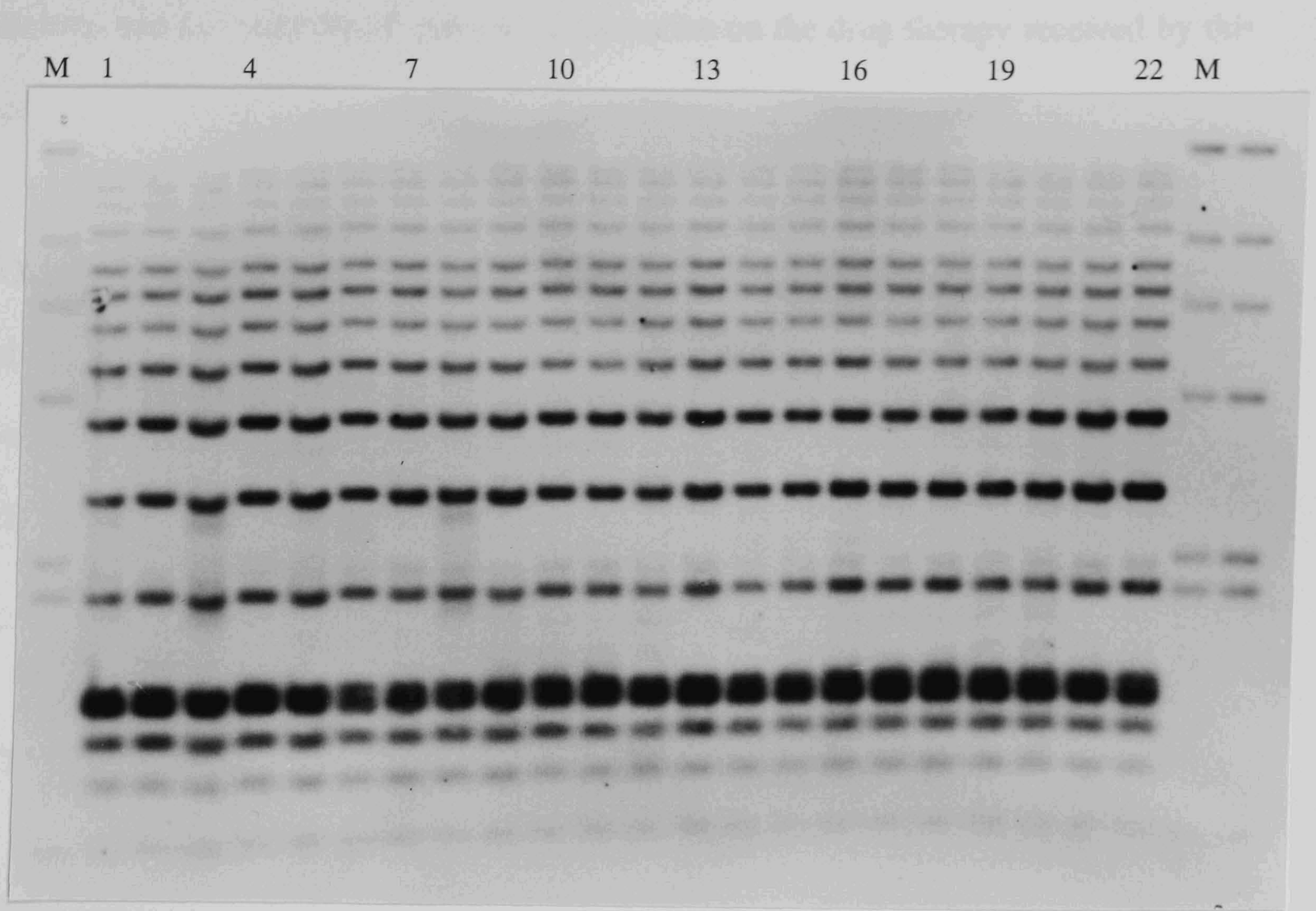


Plate 2

Internal molecular weight markers, on the same membrane as shown in Plate 1.

The IS6110 probe was removed by treatment with sodium hydroxide and SDS, then a second hybridisation with probes to the internal molecular weight markers applied to each sample of mycobacterial DNA (*Pvu* II-digested supercoiled DNA ladder and Phi X 174-*Hae* III) was carried out.

M: lambda *Hind* III (external) molecular weight marker

isolates had the same RFLP pattern. Information on the drug therapy received by this patient was not available however.

Overall, a high degree of heterogeneity was observed between strains isolated from different patients (Figure 3.1.2). However, four clusters of cases were observed in which indistinguishable strains were isolated from between two and five patients. These are summarised in Table 3.1.6.

Table 3.1.6 Clusters of cases with indistinguishable strains of *M. tuberculosis* on RFLP analysis.

Patient	Age/Sex	Source lab.	Positive specimens	Date referred
A	23 M	UCH	Bronchial washing, urine	July - August 1992
B	24 F	UCH	Sputum, pleural fluid, blood	Sept. - Oct. 1992
C	42 M	UCH	Blood	November 1992
D	41 M	Charing Cross	Sputum, bronchial washing, urine	March 1993
E	24 M	UCH	Pleural fluid	August 1993
F	32 M	N. Middlesex	Lymph node	December 1991
G	NS F	N. Middlesex	Urine	March 1992
H	34 M	Westminster	Lymph node	July 1992
I	46 M	Royal London	Lymph node, sputum	July 1992 - March 93
J	30 M	Royal London	Sputum	October 1993
K	27 M	Westminster	Sputum	November 1993
L	NS M	Royal Free	Sputum	December 1993

NS: not stated

Four clusters of cases were detected:

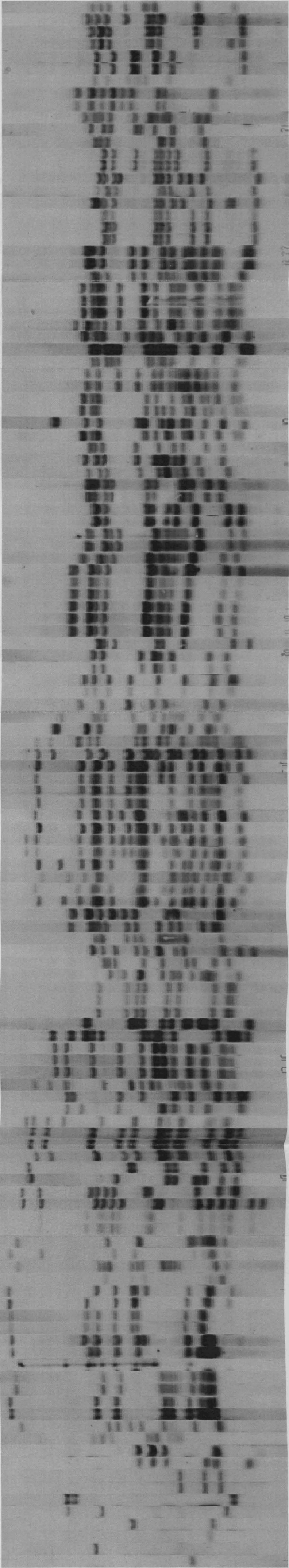
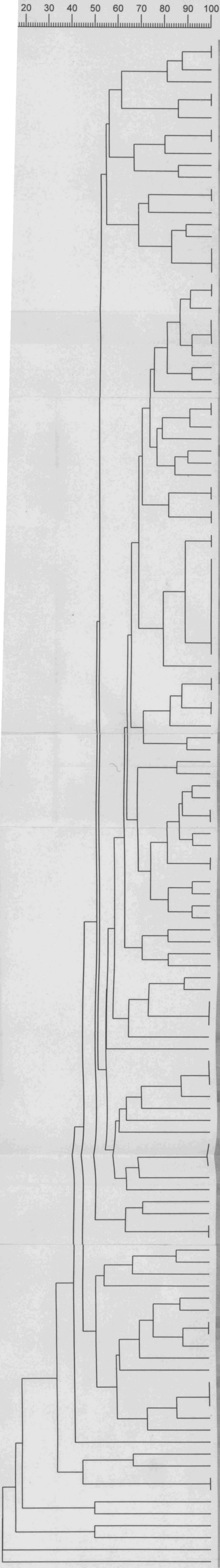
patients A - E: 10 bands (IS6110 copies)

patients F - G: 14 bands

patients H - I: 8 bands

patients J - L: 12 bands

All isolates were susceptible to rifampicin, isoniazid, pyrazinamide, ethambutol and streptomycin, except the second isolate from patient I which had acquired resistance to pyrazinamide.



rktb1 15
rktb2 7
rktb12 12
rktb12 18
rktb12 20
rktb12 21
rktb12 6
rktb1 22
rktb1 23
rktb2 21
rktb3 22
rktb12 17
rktb13 6
rktb13 7
rktb13 12
rktb13 2
rktb13 8
rktb13 9
rktb13 10
rktb13 11
rktb3 23
rktb3 24
rktb1 13
rktb6 13
rktb6 14
rktb6 15
rktb12 19
rktb2 20
rktb10 4
rktb6 6
rktb1 20
rktb1 21
rktb6 16
rktb3 8
rktb3 20
rktb6 10
rktb3 6
rktb3 25
rktb12 2
rktb2 6
rktb2 10
rktb2 9
rktb2 11
rktb1 2
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rktb3 4
rktb3 13
rktb3 14
rktb3 15
rktb13 13
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rktb6 7
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rktb10 12
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rktb1 17
rktb12 14
rktb12 11
rktb6 20
rktb10 5
rktb10 8
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rktb3 12
rktb10 9
rktb1 5
rktb1 7
rktb2 3
rktb2 19
rktb13 17
rktb13 18
rktb10 10
rktb13 14
rktb12 5
rktb12 10
rktb13 16
rktb3 7

Figure 3.1.2

Gelcompar analysis of IS6110 RFLP patterns for *M. tuberculosis* isolates from HIV seropositive patients.

The isolates have been sorted according to degree of similarity, shown by a dendrogram. A reconstructed gelstrip is shown next to each isolate.

RFLP analysis was repeated in parallel on strains from patients A to D, and confirmed the initial findings (Plate 3). Patient E, who belonged to the control group, was only recognised as part of the same cluster at a later stage.

Further clinical and epidemiological information on patients F to L involved in the three smaller clusters was not available as these patients had been diagnosed and treated at genitourinary medicine clinics. However, permission was obtained to examine the case histories of the patients identified in the first, larger cluster from the clinicians responsible for their care.

Characteristics of a cluster of patients with indistinguishable strains

The index case, patient A, was a 23 year old man who had recently arrived in the United Kingdom from Brazil, and presented in June 1992 with productive cough, fever and weight loss. He was admitted with a clinical diagnosis of right lower lobe pneumonia and treated conventionally. An HIV antibody test was positive and his CD4 lymphocyte count was 100 per μl . When he had not responded to treatment a week later he was transferred to Ward X of an HIV inpatient facility at Hospital 1. The following day he had a bronchoscopy and bronchoalveolar lavage performed on adjacent ward Y in a dedicated area separated from the main ward by a door. At that time this area had an extractor fan that vented separately from the main ward, but not negative pressure ventilation. Bronchoalveolar lavage fluid and sputum were positive for acid fast bacilli and so he was isolated in a cubicle for 14 days and started on

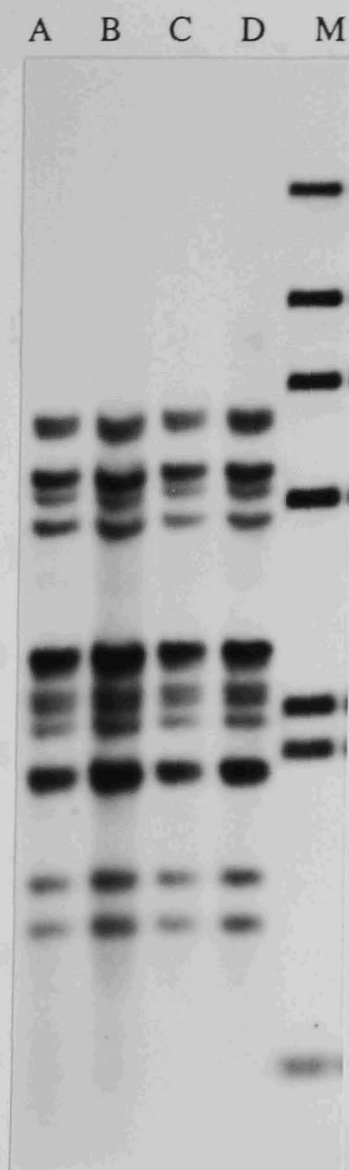


Plate 3 A cluster of cases with indistinguishable strains.

IS6110 RFLP analysis was repeated on DNA samples extracted from the *M. tuberculosis* isolates from patients A to D (Table 3.1.6).

Patient E, not known to be HIV seropositive, was a member of the control group and was only recognised as part of this cluster at a later stage.

M: lambda *Hind* III molecular weight marker

anti-tuberculous therapy with rifampicin, isoniazid, ethambutol and pyrazinamide several hours after bronchoscopy. *M. tuberculosis* fully susceptible to these drugs was isolated from the samples. After three weeks he had improved and was transferred to a convalescence unit. At follow-up after nine months therapy he had fully recovered.

Patient B was a 24 year old East African woman, found to be HIV antibody positive two years previously, with a CD4 count of 30 cells per μl . In April 1992 she complained of fevers, sweats, weight loss and cough. Respiratory and microbiological investigations were negative and she improved without systemic treatment. In June 1992 she was admitted to ward Y, one day prior to patient A, because of retinal branch vein occlusion. Her chest X-ray at that time was normal and she was discharged after one month. Eight weeks later she was readmitted to ward Y with a short history of dry cough, diarrhoea, vomiting, anorexia and hepatomegaly. Chest X-ray showed an enlarged right hilum and patchy shadowing in the right lower zone. A right pleural effusion developed. Bronchoscopy and liver biopsy were performed and acid fast bacilli were seen in both bronchoalveolar lavage fluid and liver tissue, whereupon the patient was isolated and treated with rifampicin, amikacin, ciprofloxacin and ethambutol. This was changed to rifampicin, isoniazid and pyrazinamide when fully susceptible *M. tuberculosis* was cultured. Although her tuberculosis responded to treatment, the patient died from other HIV-related problems eight months later. A post-mortem examination was not performed.

Patient C was a 42 year old Caucasian man, HIV seropositive since 1986, with a previous history of *Pneumocystis carinii* pneumonia (PCP). He was admitted to

ward Y of Hospital 1 in May 1992 because of cough and breathlessness. A bronchoscopy was normal and his CD4 count at that time was 30 cells per μl . In June 1992 he was readmitted to ward Y for investigation of weight loss and cough and he was there until the day following patient A's admission. A chest X-ray was consistent with past PCP but other respiratory and microbiological investigations were normal. Cytomegalovirus retinitis was diagnosed and a Hickman line inserted. He was readmitted in July 1992 because of neutropenia and *Pseudomonas aeruginosa* bacteraemia, related to infection of the Hickman line. A month later he became severely unwell with fever, hypotension and breathlessness and was transferred to the intensive therapy unit where he died three days later. At post-mortem, oesophagitis, shock lung and CMV colitis were found, in addition to a mediastinal lymph node, histological examination of which revealed granulomata with acid fast bacilli. *M. tuberculosis* was isolated from blood and urine collected shortly before the patient's death.

The fourth patient, D, was a 41 year old man, HIV seropositive since 1985, who was seen in the HIV clinic of Hospital 2 in February 1993 with a six week history of cough, shortness of breath, fever, night sweats and weight loss. Shadowing in the right mid zone was observed on chest X-ray, and at bronchoscopy, acid fast bacilli were seen in bronchoalveolar lavage fluid. *M. tuberculosis* fully susceptible to first line antituberculous drugs was subsequently isolated from his lavage fluid, sputum and urine. He was commenced on standard antituberculous drug therapy with an adequate response. The patient lived alone and had no known connection with any of the other cases or with Hospital 1.

Case E was a 24 year old man who worked as a staff nurse in Hospital 1. In August 1993 he presented with a short history of fever, cough and shortness of breath. A pleural effusion was detected on examination, culture of which yielded *M. tuberculosis*. This man, who was in good health prior to this episode, had no known risk factors for HIV infection and was not advised to be tested for HIV antibody by the clinicians managing his case. At the time of his illness he worked on the adolescent oncology unit, which is less than 100 metres from Ward Y across a courtyard. Patients from this unit sometimes received nebulised pentamidine on Ward Y and were escorted there by nurses. However, Case E subsequently left employment at Hospital 1 and it is not known whether he actually performed this duty. Nor are records available from the nursing school to show whether he had a previous attachment as a student nurse on the HIV unit.

In summary, five patients with strains of *M. tuberculosis*, indistinguishable on the basis of RFLP typing and antimicrobial susceptibility were detected. Patients A, B and C overlapped in their admission to the same hospital ward (Figure 3.1.3). Case A presented with open pulmonary tuberculosis first, whereas cases B and C, both of whom had very low CD4 lymphocyte counts, subsequently developed disseminated tuberculosis within several weeks. Case E was a healthcare worker who worked on a unit close to this ward and developed tuberculosis nearly 12 months later. No epidemiological link was established between these cases and patient D.

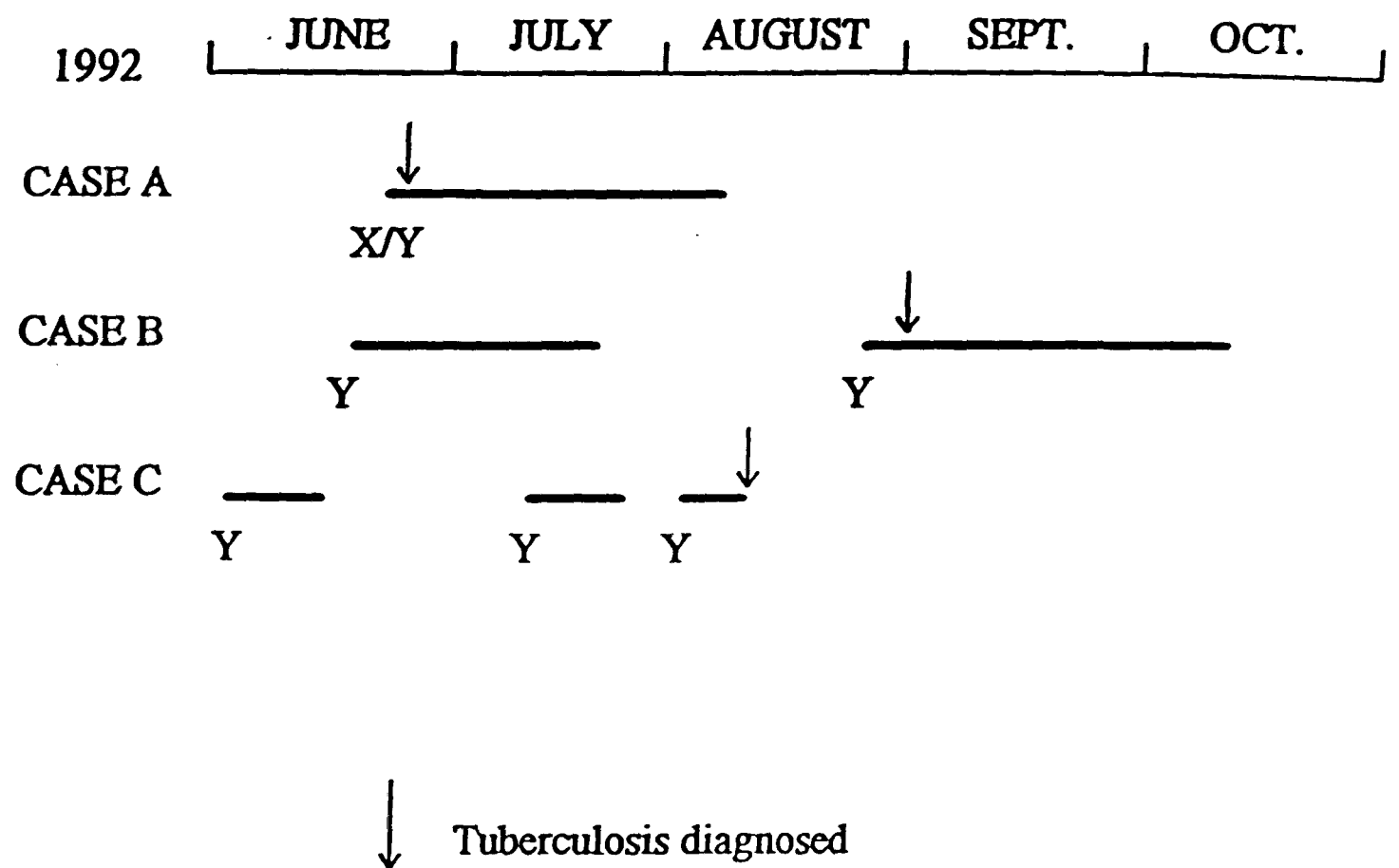


Figure 3.1.3

A cluster of cases with indistinguishable strains

Overlap of cases A to C on wards X and Y in hospital 1.

Control patients

Isolates of *M. tuberculosis* from patients not known to be HIV positive were heterogeneous with regard to RFLP pattern (Figure 3.1.4). Apart from multiple isolates from the same patient, which were indistinguishable by eye or Gelcompar analysis, only two pairs of similar isolates from different patients were discovered. However, these strains had only two and three copies of IS6110 respectively, so that RFLP analysis using a probe to this insertion sequence may not be adequate to distinguish between such strains if they are genuinely unrelated. The patients involved in these two pairs attended different hospitals.

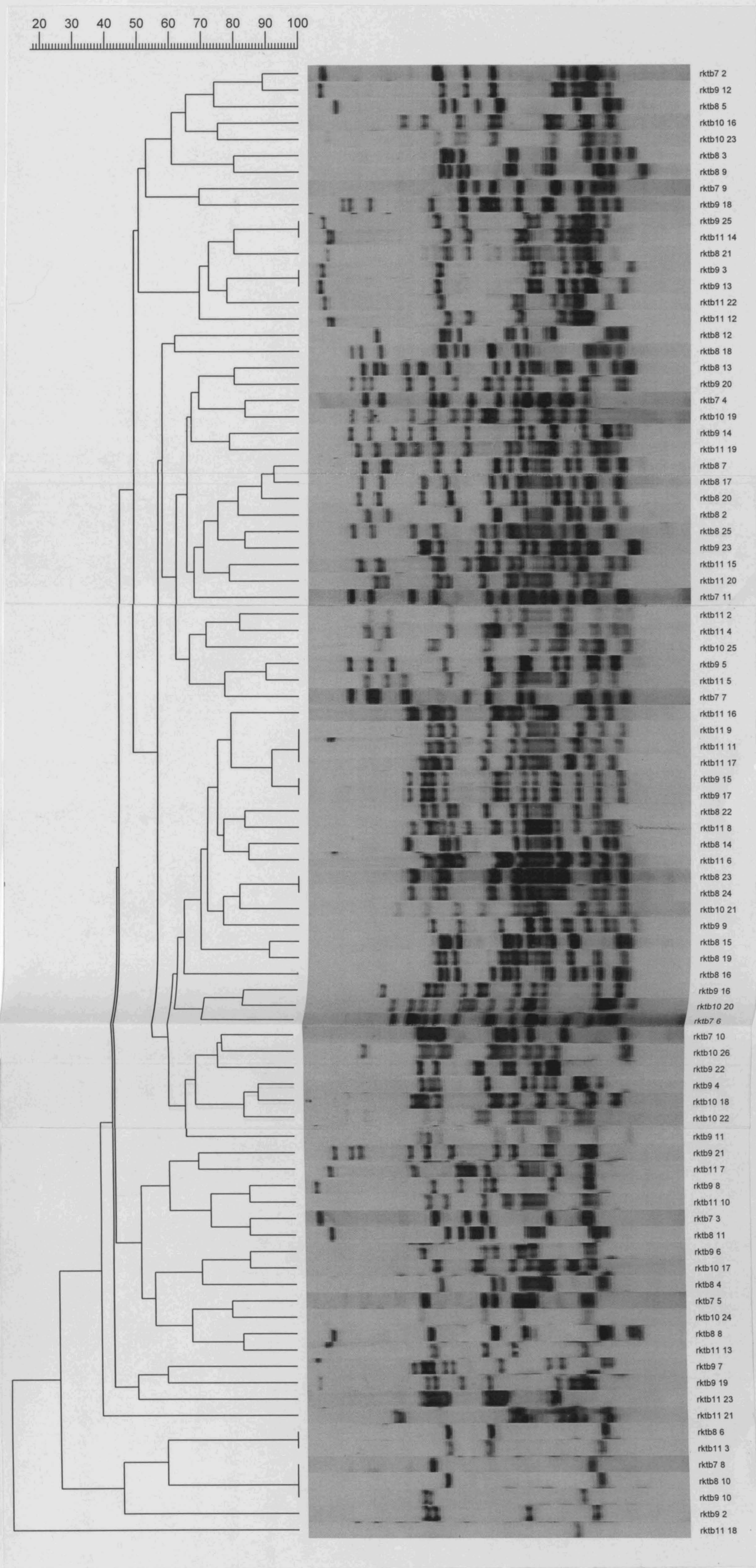


Figure 3.1.4

Gelcompar analysis of IS6110 RFLP patterns for *M. tuberculosis* isolates from control patients.

The isolates have been sorted according to degree of similarity, shown by a dendrogram. A reconstructed gelstrip is shown next to each isolate.

3.2 *MYCOBACTERIUM AVIUM* COMPLEX

56 AIDS patients with disseminated *M. avium* complex (MAC) infection were studied. All had multiple isolates of MAC, including at least one from a blood culture or bone marrow biopsy, sent between December 1991 and June 1994. The isolates from these patients were referred from seven hospitals: Westminster/Chelsea and Westminster Hospital (35 patients), University College Hospital (7), King's College Hospital (7), Royal Sussex County Hospital, Brighton (3), Royal Free Hospital (2), St. Bartholomew's Hospital (1) and St. George's Hospital (1). 53 of the patients were male with a median age of 36 (standard error 7.0) years. Altogether, 210 isolates were examined; almost all were viable after storage on Lowenstein-Jensen slopes at room temperature for up to two years. 178 of these yielded sufficient DNA after embedding the cells in agarose plugs to give adequate patterns on PFGE. The breakdown of isolates in terms of the clinical specimens from which they were cultured is given in Tables 3.2.1 and 3.2.2. Results were obtained for a median of three isolates per patient (range 2 to 8).

Table 3.2.1 Clinical specimens from which MAC isolates used in this study were obtained.

Clinical specimen	Number of specimens
Blood cultures and bone marrow	116
Sputum and bronchial washings	31
Faeces	18
Urine	2
Pleural aspirate	2
Lymph node	3
Liver	2
Other	4
Total	178

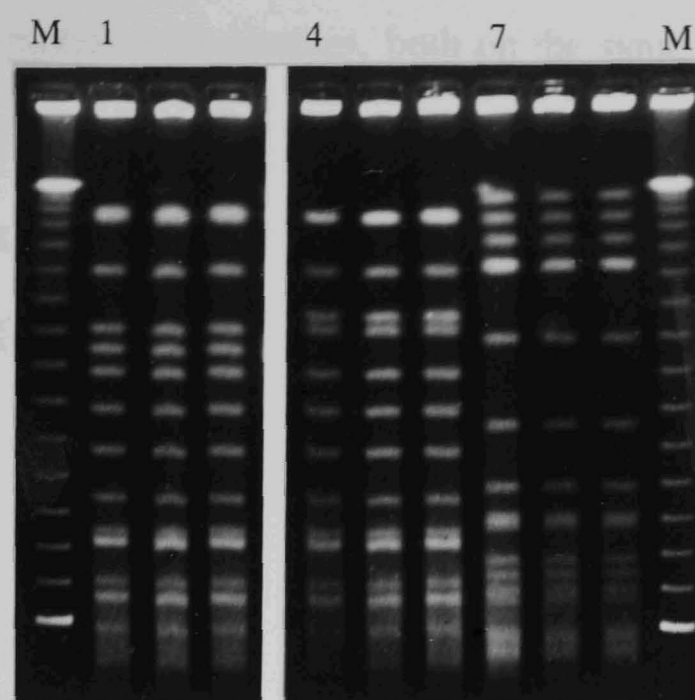
Table 3.2.2 Sites of MAC infection/colonisation among the patients in this study.

Positive clinical specimen	Number of patients
Blood/bone marrow only	13
Blood/bone marrow plus:	
sputum/bronchial washing	23
faeces	10
sputum/bronchial washing and faeces	6
other*	4
Total	56

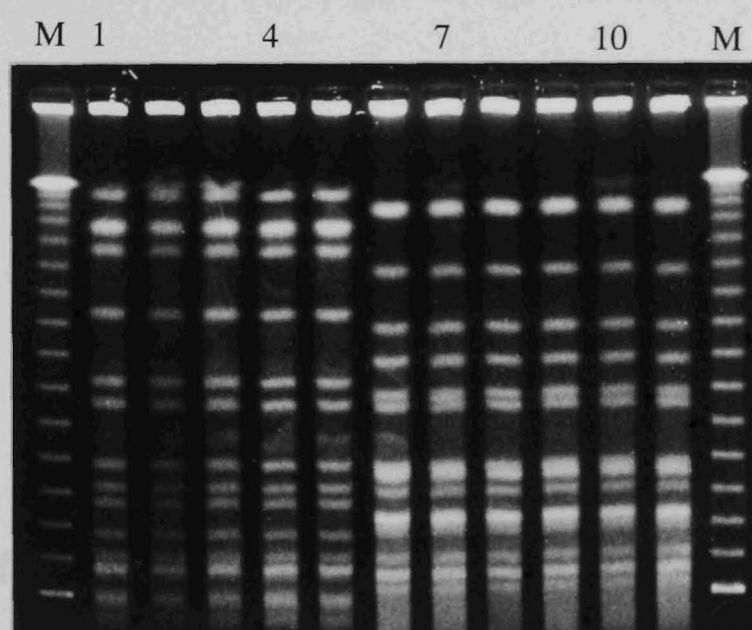
* lymph node, liver, urine, pleural aspirate

Preliminary experiments were carried out to optimise the yield of mycobacterial DNA and the efficiency of restriction digestion prior to pulsed-field gel electrophoresis. Difficulty was experienced initially in extracting sufficient DNA from the cells, probably due to the lysis-resistant nature of the mycobacterial cell wall. Treatment of the harvested cells with the detergent Triton-X-100 as recommended by Charvin *et al.* (1991), or increasing the concentrations of lysozyme (to 2 mg/ml) and proteinase K (to 1 mg/ml) used for cell lysis, or prolonging the incubation with these enzymes had no effect on DNA yield (results not shown). The problem was overcome by increasing the number of cells in each plug: resuspending each 10 ml culture in 200 μ l buffer. Since this work was completed, some improvement in DNA yield has been achieved with a variation of the method of Slutsky *et al.* (1994) by incorporating 0.5 M sucrose in the culture broth and resuspension medium, and by adding ampicillin, D-cycloserine and D-threonine to the broth prior to harvesting (M. Hunnab, personal communication). The concentration of restriction enzyme around agarose plugs containing mycobacterial DNA was critical for efficient digestion. A small volume (100 μ l) of buffer containing enzyme gave better results than the same number of units of enzyme in a larger volume at a lower concentration.

Restriction digestion of chromosomal DNA with the enzyme *Ase* I resulted in a median of 11 fragments (range 6 to 17) per isolate in the size range 50 to 700 kilobases. This gave rise to patterns which were easy to read and were highly polymorphic, giving satisfactory discriminative power for comparing the patterns of different strains (Plate 4). The large restriction fragment (LRF) patterns obtained were reproducible from gel to gel under the same electrophoretic conditions.



A



B

Plate 4 *Ase* I large restriction fragments of *M. avium* complex genomic DNA separated by PFGE.

Isolates from five patients, each with one strain of MAC throughout, which differs from that of the other patients.

Lanes	Patient	Isolates from:	Date
A 1-3	JM	sputum, bone marrow, blood	Oct. 93
A 4-6	CC	liver, blood, sputum	July-Oct. 93
A 7-9	AY	bone marrow, sputum, blood	Sept.-Oct. 93
B 1-5	WE	stool, blood, lymph node, blood (x2)	Oct. 92-June 93
B 6-11	IS	blood, sputum, stool, blood, urine, stool	Dec. 92-June 93

M: molecular weight marker - lambda phage concatamers (48.5, 97, 143.5, 194, 242.5,....1018.5 kilobases)

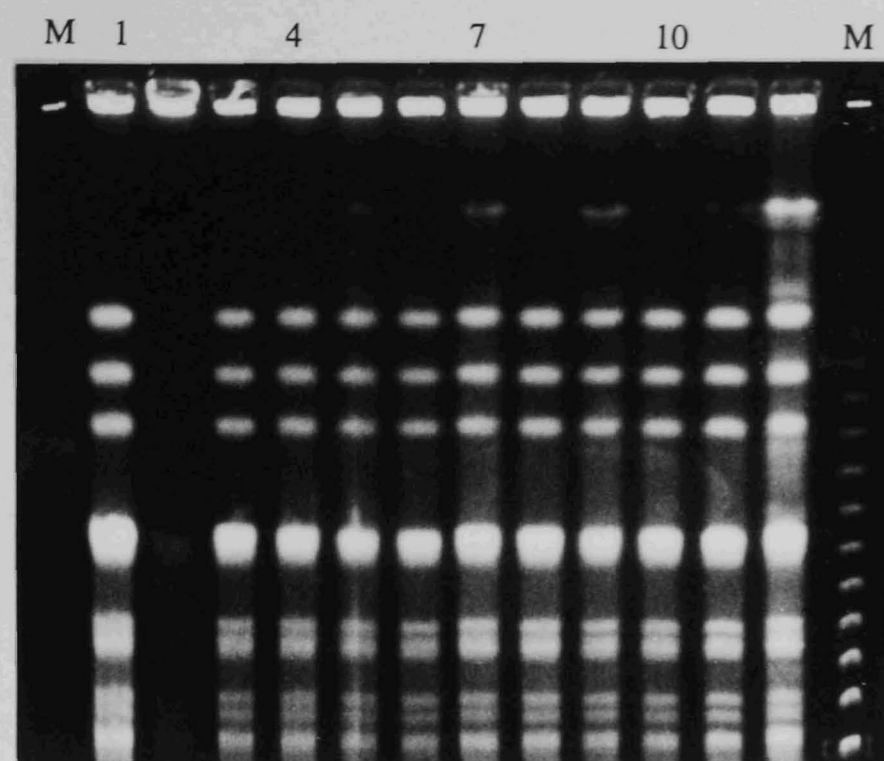
Comparison of one pattern with another, both on the same gel and between different gels, was facilitated by use of the same external markers (lambda phage concatamers) on every gel. DNA migration from track to track across each gel was found to be uniform with the CHEF DRII apparatus used, with little distortion at the ends of the gels.

In 49 of 56 patients (87.5%) all isolates obtained from the same patient had indistinguishable LRF patterns. This applied both to isolates from different clinical sites and also to those isolated at different times. The interval between receipt of isolates for individual patients varied from 7 days to 30 months; in 7 cases intervals of 12 months or more between isolates with indistinguishable LRF patterns were observed.

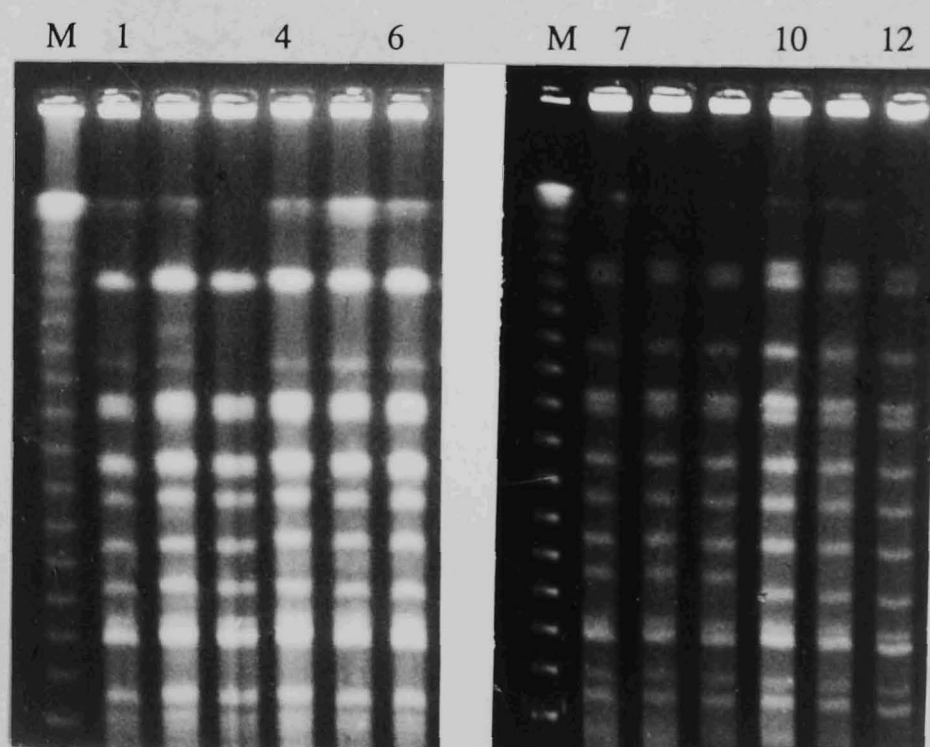
The other 7 out of 56 (12.5%) patients were each infected or colonised with more than one strain of MAC. Two patients had blood culture isolates which were different to those cultured from other sites; three had strains from stool samples which differed from those isolated elsewhere; one patient had both a blood and a stool strain which were different to others; and one patient had a sputum isolate which was distinct from his blood isolate. In each case, the patients with multiple strains from blood cultures had consecutive infections with different strains, separated by intervals of between 11 and 21 months.

Although the MAC strains isolated from most patients were unique to themselves, two clusters of cases were discovered, one of eight patients and the other of four, in which isolates with very similar LRF patterns were found in each case (Table 3.2.3, Plate 5). When these strains were analysed with a different restriction enzyme, *Xba* I, the same relationships were preserved (Plate 6). Initially five cases were recognised in the first cluster. Because most of them had been referred from Westminster Hospital during the second half of 1992, it was decided to examine all the MAC strains from HIV patients referred from this laboratory between June and December 1992. This served to include patients from whom MAC was isolated on only one occasion, or from sites which did not include blood or bone marrow (and were therefore not included in the main study). 22 further patients were identified, of whom three had a strain indistinguishable to that of the common strain. A limited amount of information on these patients was disclosed by clinicians involved with their care.

Six patients in the larger cluster had been inpatients at Westminster Hospital at various times during 1992/3 and had also attended one or both of two units in London which provide convalescence or terminal care to patients with HIV-related disease. In addition, each of these patients had received chemotherapy for malignancy or retinitis due to cytomegalovirus during the course of their illness. They were all registered at different home addresses.



A



B

Plate 5 *Ase* I large restriction fragments of *M. avium* complex genomic DNA separated by PFGE.

A Isolates from patients 3 (lanes 1-3), 8 (lanes 4-6), 4 (lanes 7-9), and 2 (lanes 10-12) in Table 3.2.3, showing part of a cluster of cases with indistinguishable strains.

B Isolates from patients I (lanes 1-3), II (lanes 4-6), III (lanes 7-9) and IV (lanes 10-12) in Table 3.2.3, showing strains with similar large restriction fragment patterns.

M: molecular weight marker (48.5 kilobase concatamers of lambda phage).

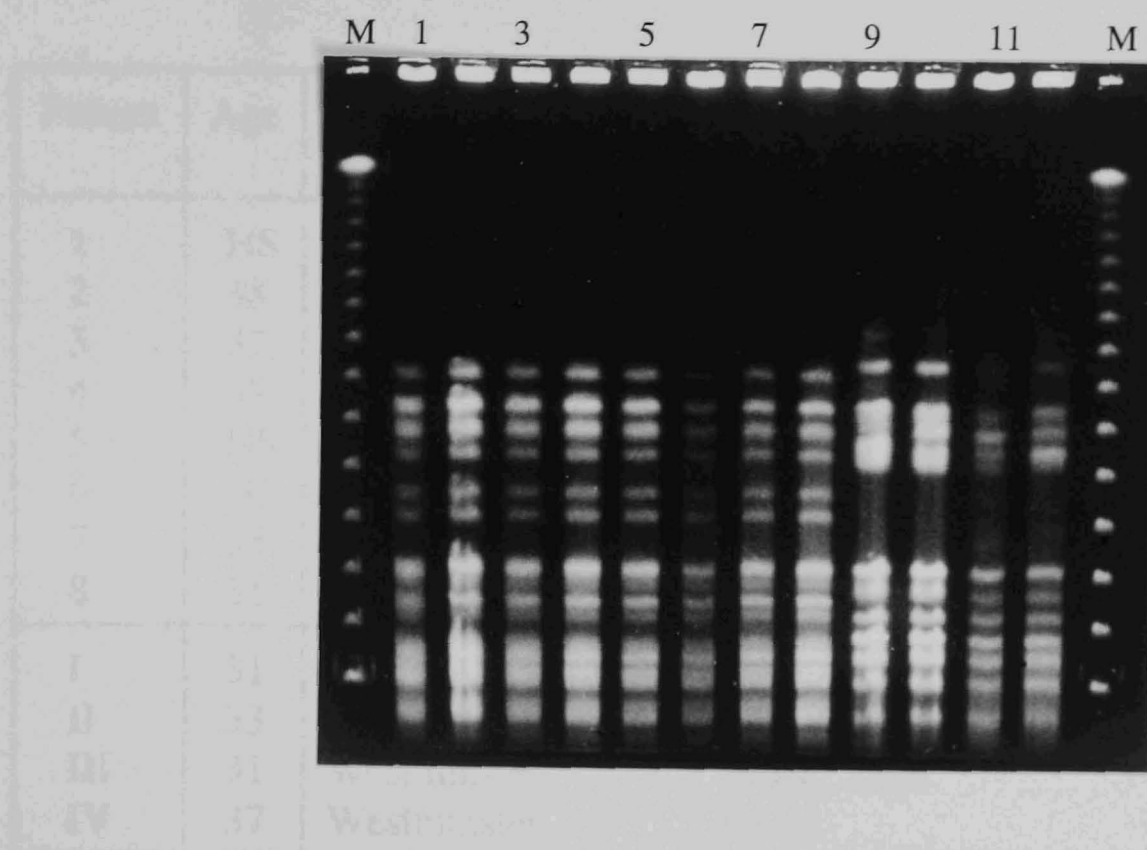


Plate 6 *Xba* I large restriction fragment patterns of MAC genomic DNA separated by PFGE.

Lanes 1 to 8: isolates from patients 1 - 8 in Table 3.2.3

Lanes 9 to 12: isolates from patients I - IV in Table 3.2.3

This confirms the relatedness of the strains shown in Plate 5.

M: molecular weight marker (48.5 kilobase concatamers of lambda phage).

Table 3.2.3 Summary of patients whose strains of *M. avium* complex were clustered by PFGE of large restriction fragments.

Patient	Age	Source laboratory	Site(s)	Date referred
1	NS	Westminster	Blood	6/92
2	38	Westminster	Blood, stool	7/92-10/92
3	39	King's College	Blood, bone marrow, sputum	10/92-4/93
4	37	Westminster	Blood, sputum	11/92-12/92
5	NS	Westminster	Blood	12/92
6	NS	Westminster	Blood	12/92
7	55	University College	Blood*	1/93
8	31	Westminster	Blood, sputum, stool	1/94-2/94
I	31	King's College	Blood, sputum	11/93-5/94
II	33	King's College	Blood	3/94-5/94
III	31	Westminster	Blood, stool	7/91-2/94
IV	37	Westminster	Blood	12/93-6/94

* other, unrelated strains were also isolated from this patient between October and December 1993.

NS: not stated

Patients I and II in the second cluster were acquaintances who had both been treated at King's College Hospital for AIDS-related diseases at the same time. The PFGE patterns of isolates from patients III and IV closely resembled but were not identical to those of the other two on *Ase* I restriction digestion, although appeared to be the same on *Xba* I digestion.

4. DISCUSSION

4.1 *MYCOBACTERIUM TUBERCULOSIS*

At the time of the study, the Regional Tuberculosis Centre at Dulwich (now the PHLS Mycobacterium Reference Unit) received mycobacteria for identification and susceptibility testing isolated at over 70 client laboratories in South East England, making up about 95% of all isolates in the region. These laboratories included those serving all but one of the large HIV units in London. The unit not covered (St. Mary's Hospital) referred its isolates during the study period to a reference laboratory in Cardiff where strains of *M. tuberculosis* were not routinely stored, and therefore were not available for typing. Nevertheless, isolates from 24 other laboratories referred between 1991 and 1994 were examined, so the collection is as representative as possible of the *M. tuberculosis* strains causing infection in HIV patients in South East England at that time.

In spite of the use of enrichment culture in liquid medium, the small proportion (11%) of strains of *M. tuberculosis* which were still alive after more than two years storage on Lowenstein-Jensen slopes at room temperature was disappointing and limited the number of strains and the time period covered by this retrospective study. Similar findings have been made subsequently by others using strains from the same collection (L. McKnight, S. Goss, unpublished observations). As a result, *M. tuberculosis* isolates are now stored on Lowenstein-Jensen slopes at -20°C in the laboratory. Reference collections elsewhere that are stored in this way have remained viable for many years. A typing method involving a polymerase chain reaction to amplify very

small amounts of DNA from the cells of non-viable strains or those giving an inadequate yield in the initial DNA preparation step might overcome this problem. At the time of the study, no single PCR-based method was generally accepted as consistently reliable. Two techniques published subsequently which might be suitable however, are spoligotyping of the spacers in the direct repeat region of the *M. tuberculosis* chromosome (Kamerbeek *et al.* 1994), and heminested inverse PCR (Patel *et al.* 1996). The latter has been attempted with stored strains isolated from tuberculosis patients in London during 1993 as part of a PHLS study.

Information on the HIV status of the patients from whom the strains were isolated was provided by the referring laboratories. However, although patients with tuberculosis in the control group were not known to be HIV seropositive, testing for HIV antibody was not necessarily carried out, nor were risk factors for HIV infection ascertained. It is therefore possible that some patients in this group may have been HIV positive but not recognised as such. Although in theory the names of control patients could be matched with those on the CDSC HIV database, information in the latter is recorded in the form of date of birth and Soundex name code (a one-way coding system), so this approach would still not enable all HIV seropositive 'controls' to be excluded with certainty.

The median age of patients in HIV and control groups was similar, although the range of ages was narrower for the HIV group, a higher proportion of which was male. This reflects the predominance of young adult males in the HIV population of South East England at present. Although used previously (Yates *et al.* 1993), the accuracy

of inferring ethnic origin from surnames is very limited. For example, persons of Caribbean or South American origin often have 'European' surnames, and surname may be changed by marriage. A more reliable approach was used for the United Kingdom 1991 Census in which people were asked about their ethnic group for the first time. The Census provided much new data about ethnic minority groups in Britain (A. Pozniak, personal communication), but its methods are not applicable to this study. Nevertheless, it was observed that HIV seropositive tuberculosis patients were much more frequently of presumed African origin than from the Indian subcontinent, whereas the proportions of control patients in these two ethnic groups were similar (Table 3.1.3). This is probably because HIV infection is more common among African than among Asian people living in the region at present.

Comparison of the sites of tuberculous infection in the two groups showed that although pulmonary disease was the most common form in both, extrapulmonary tuberculosis was much more frequent among HIV patients. This finding is in agreement with most other studies of tuberculosis in HIV infection (Barnes *et al.* 1991, 1993, Jones *et al.* 1993, Given *et al.* 1994). Isolation of *M. tuberculosis* from blood cultures or bone marrow aspirates occurred only in the HIV group. Although disseminated tuberculosis and mycobacteraemia are more frequent in HIV infection than in immunocompetent persons, such cultures are also more likely to be taken from HIV patients as part of the investigation of fever and weight loss, common in late HIV disease. Positive stool cultures and gastric washings were also more common in the HIV group, again possibly because such specimens are routinely submitted to some laboratories as part of the investigation of other mycobacterial disease in HIV

patients such as disseminated *M. avium* complex infection. In most of these cases sputum isolates were also referred, suggesting that the main focus of infection was pulmonary rather than the gastrointestinal tract. *M. tuberculosis* was also isolated from urine samples more often in HIV patients than in the controls. This may reflect the greater likelihood of disseminated infection in the former rather than disease of the genitourinary tract, although full clinical details of most cases were not investigated.

Most isolates from HIV patients were fully sensitive to the first line antituberculous drugs tested, although the proportion of these patients with an isolate resistant to one or more drug (20/96, 20.6%) was higher than among the control group (7/84, 8.4%). It was also higher than that reported for HIV seropositive patients whose strains were referred to the Regional Tuberculosis Centre at Dulwich between 1984 and 1992 (Yates *et al.* 1993). During that period, 9 out of 162 (5.6%) patients had strains resistant to one or more of the tested drugs, of which two thirds were of presumed African ethnic origin. This was not significantly different from the rate of drug resistance observed in the overall populations (Yates *et al.* 1993). Data on drug resistance in *M. tuberculosis* isolated from HIV seropositive and seronegative patients at St. Mary's Hospital, London between 1987 and 1993 have also been published (Taylor *et al.* 1995). In that series, 7.6% of 358 cases were resistant to rifampicin and/or isoniazid, but almost all were from presumed HIV seronegative patients; no increase in drug resistant isolates was observed over the period. Nevertheless, in spite of the small increase in drug resistance observed in the present study strains resistant to both rifampicin and isoniazid (i.e. multidrug resistant tuberculosis, MDRTB) were found in only two HIV patients. Both strains, isolated from sputum in

one case and from urine in the other, were referred in 1994 from different hospitals and had different RFLP patterns. In contrast, MDRTB affecting HIV patients was widespread in the United States by 1991 (Snider & Roper 1992, Riley 1993, Haas & Des Prez 1994) and had emerged in Italy at that time (Monno *et al.* 1991, Riley 1993).

This project involved setting up the equipment and protocols required for DNA fingerprinting of *M. tuberculosis* in a clinical laboratory used to handling large numbers of mycobacteria, but which compared different strains only on the basis of phenotypic characteristics. The molecular techniques were used successfully, and have subsequently been taught to other staff in the laboratory, who have used them to investigate other collections of *M. tuberculosis* isolates. These include isolates from a nosocomial outbreak of MDRTB at Chelsea and Westminster Hospital, described below (M. Yates & R. Kent), isolates from tuberculosis patients in East London during 1993-4 (L. McKnight), and isolates collected in a study in Nepal (E. Burke).

The typing system employed, RFLP based on the insertion sequence IS6110, was valid for the strains tested in this study because all contained at least one copy of IS6110 (100% typability), the patterns obtained were reproducible when multiple strains from the same patient were typed or when the same strain was analysed on different occasions, and the discriminative power of the typing method was high. The latter is determined by the average number of copies of the repetitive element and the degree of polymorphism of their position for the strains tested; both factors were high in this study. RFLP analysis using IS6110 is most useful for isolates with unique

fingerprints and for those in clusters which contain six or more copies of the insertion sequence (Chaves *et al.* 1996). These considerations applied to all the isolates from HIV seropositive patients. However, the two pairs of isolates containing two and three copies of IS6110, from patients in the control group, require a secondary probe in order to determine whether or not they are genuinely identical. A probe to the Direct Repeat sequence, or the polymorphic GC-rich repetitive sequence, or both, would be suitable in this regard. Studies in which a significant proportion of isolates possessed between two and five IS6110 copies have successfully used probes to these repetitive elements to subdivide clusters (Alland *et al.* 1994, Chaves *et al.* 1996, Strassle *et al.* 1997).

A limitation of using a single probe in molecular epidemiological work is the difficulty in interpreting the relationship between strains with very similar but not identical fingerprint patterns. For example, strains with more than six copies of IS6110 that differ from one another by only one or two bands on hybridisation may be closely related if the difference has arisen through a transposition event on the chromosome affecting the position of one of the bands. van Soolingen *et al.* have illustrated such a case occurring in a family outbreak of tuberculosis in Czechoslovakia (van Soolingen *et al.* 1991). However, analysis of sets of similar isolates with pTBN12 shows that while most have indistinguishable patterns with this probe, there are exceptions (Chaves *et al.* 1996). Strains with very similar but different IS6110 patterns cannot necessarily be included in the same cluster, therefore. For this reason, isolates in the present study were classified as either indistinguishable or not. Although analysis of the RFLP patterns with GelCompar software generated a

dendrogram showing apparent degrees of relatedness between isolates, it should be noted that this is only a mathematical calculation and does not imply phylogenetic or epidemiological relationships between them. Use of the Gelcompar system was not altogether straightforward. For example, it sometimes separated isolates from the same patient that appeared indistinguishable by eye. This was usually the case when isolates were on different gels, run to slightly different distances, even though the normalisation step of the analysis was supposed to take such factors into account. The usefulness of Gelcompar depends on the number of isolates to be compared: for a small number, comparison by visual inspection is probably satisfactory, whereas computer assistance is required when there are many isolates. The best approach was found to be an initial computer analysis to sort the isolates according to degree of relatedness, followed by visual inspection of those isolates clustered together to confirm or refute the computer assessment.

For 25 HIV patients who had multiple isolates of *M. tuberculosis* the results of IS6110 RFLP analysis were consistent in each case with infection due to a single strain. In some patients, infection with one strain at multiple sites was due to disseminated disease. Relapse of infection with the same strain was less likely in most cases because of the short time intervals between receipt of these multiple isolates. No evidence for infection with more than one strain per patient (re-infection) was obtained. The RFLP data also show that in one patient the development of pyrazinamide resistance was consistent with acquisition of resistance by the original strain and not to re-infection with a different strain. It should be noted however that

susceptibility testing of *M. tuberculosis* to pyrazinamide is much less straightforward than for other antituberculous drugs (section 2.1.2). False resistance to pyrazinamide is sometimes seen, but tests on isolates appearing resistant are repeated. Strassle *et al.* (1997) studied a cohort of 46 HIV patients with tuberculosis in Zurich, Switzerland, and also found that among the patients from whom multiple isolates were available, the IS6110 patterns remained virtually stable over a period of up to four years as well as during the emergence of drug resistance. No evidence for reinfection of a patient with a different strain was obtained.

Comparison of the DNA fingerprint patterns obtained by IS6110 RFLP analysis showed considerable heterogeneity among the strains isolated from HIV seropositive patients. 12 of the 96 patients belonged to one of four clusters of indistinguishable strains. From these figures, 8 patients (8.2%) may have acquired tuberculosis from recent person-to-person transmission rather than by the reactivation of latent infection. This proportion is significantly higher than in the control group, where no clusters of cases were found, if strains containing three or fewer copies of IS6110 are excluded from analysis ($p < 0.001$ in chi-squared test with Yate's correction).

Nevertheless, published studies of tuberculosis transmission in some other countries suggest that the proportion of cases due to recently acquired infection is higher than this: at least a quarter of cases in Denmark (Yang *et al.* 1995a) and a third or more in New York City (Alland *et al.* 1994) and San Francisco (Small *et al.* 1994). One explanation for the lower proportion observed here is that the patients were from a wide geographical area (South East England) and were heterogeneous with regard to

socioeconomic group and ethnic origin. About a third of HIV seropositive tuberculosis patients were of presumed African origin (Table 3.1.3); many of these may have acquired their original infection abroad before coming to live in the region. Increased patient diversity is expected to be associated with heterogeneity of infecting strains. A French study in which clinical isolates of *M. tuberculosis* from patients attending selected hospitals in Paris during 1993 were systematically typed using IS6110 RFLP analysis and other methods, supports this conclusion (Torrea *et al.* 1996). Only 13% of cases were thought to have had recently transmitted tuberculosis, but three quarters of the patients were said to be foreigners from various parts of the world including Africa and Asia.

Direct comparison of the results with other studies of tuberculosis transmission abroad, however, may be misleading because only strains from HIV seropositive patients and from selected controls were typed here, whereas most studies have examined all strains isolated in a given community over a particular time period. It is likely that clusters of cases resulting from person-to-person transmission of *M. tuberculosis* would have been unnoticed had transmission occurred from an HIV positive case to an HIV negative person not included in the study, or vice-versa. In order to avoid underestimating the extent of recent transmission in a community it is therefore necessary to type all strains isolated from patients with tuberculosis. Such a study has been set up by the PHLS Communicable Disease Surveillance Centre to examine all available strains isolated during 1993 from patients with tuberculosis in South East England, and is using some of the isolates studied here. Nevertheless, if time and resources allow only a sample of cases to be studied, then infection in HIV

positive patients is likely to be the most sensitive indicator of the spread of tuberculosis. HIV seropositivity is a clear risk factor for belonging to a cluster of cases in other studies (Hamburg & Frieden 1994), partly because of the increased susceptibility of these patients to developing clinical disease after infection and because they are frequently looked after together in special HIV units, both in and out of hospital, where aerosol-generating procedures are also performed.

The cluster of cases described in this study (patients A to E) illustrates some of these points. Cases A, B and C were epidemiologically related: patients B and C were present on ward Y of the hospital concerned at the time that patient A, who was sputum smear positive, was bronchoscoped there. At that date, the bronchoscopy room, which opened directly on to ward Y, did not have negative pressure ventilation. Although the period of time spent by patient A on ward Y was short, it included this cough-inducing procedure. In addition, the two wards X and Y are connected by an atrium, and patients from each ward use facilities on the other. The three patients had no known contact with each other apart from admission to this hospital. Taken in conjunction with the results of RFLP analysis on the strains, there is sufficient evidence to conclude that transmission of tuberculosis occurred from patient A to patients B and C (Kent *et al.* 1994, Miller *et al.* 1995). The latter were severely immunocompromised and developed pulmonary and/or disseminated tuberculosis within several weeks of exposure to the index case. Case E is probably linked to these three. Although his illness was apparent approximately 12 months later, he was a healthcare worker on a unit close to Wards X and Y in the same hospital. It is possible, although it cannot be proven, that contact with patients A, B and C may

have occurred.

The relationship between these cases and patient D is unknown however. The latter resisted attempts at contact tracing. It is possible that an unidentified intermediate patient who was HIV negative and therefore not studied, or whose strain was not referred to the laboratory, or was non-viable, may have acted as a link between the first cases and this man. Transmission could have occurred in a public place such as a bar, between individuals who did not know each other. This scenario was suggested to be the link between several cases in the community outbreaks of tuberculosis investigated in King County, Washington (Tabet *et al.* 1994) and in Berne, Switzerland (Genewein *et al.* 1993). Conventional contact tracing again failed to identify the routes of transmission in these outbreaks.

Although epidemiological information clearly links patients A to C, further molecular work using more recently described methods might be able to resolve whether patients D and E should be included in the same cluster. RFLP analysis of the direct repeat region (Torrea *et al.* 1995) and of the domains which hybridise to oligonucleotide (GTG)₅ (Warren *et al.* 1996) both appear to have the ability to subdivide clusters of strains with indistinguishable high copy IS6110 patterns, and would be suitable in this regard.

This cluster of cases would not have been recognised if isolates of *M. tuberculosis* from HIV patients had not been routinely stored in the laboratory and without the use of RFLP typing. The recognition and investigation of future outbreaks would be

greatly facilitated if all strains are typed upon referral to reference laboratories, as opposed to selected isolates as part of a retrospective investigation. Projects to do this have been set up elsewhere, including New York City and the Netherlands. On a larger scale, a concerted action to collect fingerprints from many European countries and store them in a database together with clinical and bacteriological information has been initiated (Small & van Embden 1994). It is anticipated that this will eventually be able to provide clues about the impact of, for example, HIV, migration, drug resistance and BCG vaccination on tuberculosis transmission within Europe.

The hospital outbreak of tuberculosis uncovered in this investigation was the first to be described among HIV patients in the United Kingdom. It was suggested at the time that units caring for such patients should review their procedures and policies for managing cases of suspected tuberculosis and preventing its transmission (Kent *et al.* 1994). Suitable guidelines for limiting the spread of airborne pathogens have been published by the Centers for Disease Control, Atlanta (1990, 1994). Although expensive to implement fully, there is agreement that stricter protocols to control the transmission of tuberculosis should be adopted in HIV units (Scott & Holton 1994). In spite of this however, two further outbreaks have subsequently come to light in HIV units in London. Potentially these are more serious because they involve multidrug resistant strains of *M. tuberculosis*. In one, a 33 year old Portuguese man was admitted to a six bedded bay of the HIV ward at Chelsea and Westminster Hospital with a diagnosis of *Pneumocystis carinii* pneumonia (CDSC 1995b, Hannan *et al.* 1996). At that time (April 1995) he was taking quadruple antituberculous therapy, but one month later *M. tuberculosis* resistant to rifampicin, isoniazid,

pyrazinamide, rifabutin, clofazimine and ethionamide was cultured from sputum. Four patients in the same bay as the index case developed active tuberculosis within eight weeks of exposure, and their isolates had the same susceptibility and RFLP patterns. As a result of this outbreak local guidelines for the diagnosis and management of HIV-associated MDRTB were drawn up by a committee of local clinicians (London Communicable Disease Monitor, October 25th 1995, Insert 4). The second outbreak of MDRTB, announced by the Chief Medical Officer in June 1996, occurred in at least four HIV positive patients at St. Thomas's Hospital (Calman 1996). Investigations are continuing, but several of the patients were infected with strains showing the same drug resistance and RFLP patterns. The Department of Health is currently preparing guidelines for the management of suspected cases of MDRTB.

It is regrettable, despite the extensive and widely reported experience of nosocomial transmission of tuberculosis among HIV patients in the United States and elsewhere, that many units caring for this group of patients in the United Kingdom do not enforce adequate policies and procedures to prevent transmission until after such an event has occurred. It is important that health care workers should maintain a high index of suspicion for tuberculosis in view of the frequently atypical presentation in HIV infection. Any patient with respiratory symptoms attending such a unit should be regarded as potentially having MDRTB until proved otherwise. These cases should be managed in single rooms with negative pressure ventilation and proper enforcement of isolation procedures. It is crucial that all aerosol-generating procedures such as bronchoscopy must be carried out in a separate, adequately ventilated area. Staff

should have evidence of delayed hypersensitivity to purified protein derivative before working on the unit, and staff who are themselves HIV positive should not work with these particular patients. Wherever possible, antituberculous therapy should be directly supervised to prevent the emergence of MDRTB, both in hospital and in the community.

4.2 *MYCOBACTERIUM AVIUM* COMPLEX

This study was an attempt to investigate the natural history of disseminated MAC infection in AIDS by typing isolates from patients with positive blood or bone marrow cultures. These sites were chosen because isolation of MAC from sputum or stool samples alone may reflect colonisation with the organism rather than disseminated infection. Patients with more than one positive culture were selected first to enable comparison of organisms isolated at different times or from different body sites in the same individual. This policy served to exclude patients with transient bacteraemia, who may not be representative of those with established disseminated infection (Kemper *et al.* 1994). It also reduced the likelihood of inadvertently studying false positive cultures which might have resulted from cross contamination of specimens from different patients in one of the source laboratories. Pseudo-outbreaks of MAC have been described in several laboratories (which did not provide isolates used in this study) where carry-over contamination from positive to subsequent negative culture bottles occurred in BACTEC 460 TB systems (Vannier *et al.* 1988, Bignardi *et al.* 1994, Burki *et al.* 1995). In each case this was thought to have resulted from inadequate heating of the needle used to sample each bottle between cultures; even a needle temperature of 240.7°C, below the required temperature of 250 to 300°C, was associated with carry-over contamination on one occasion (Burki *et al.* 1995).

The patients whose isolates were studied were predominantly young men. This reflects the population of patients with disseminated MAC infection seen at the main source hospitals. After blood and bone marrow cultures, MAC was isolated more

often from sputum than from stool samples in the patients selected. This is in spite of the fact that stool samples are more often cultured for MAC as part of routine investigation protocols in many HIV care centres. It may reflect the relative ease of culturing mycobacteria without contamination from sputum compared to stool samples, rather than the true frequency of MAC in the gut or respiratory tract of these patients. Many clinical laboratories employ a method designed to reduce contamination by microorganisms present in the normal flora of such specimens, for example by incubating them briefly with strong alkali before culture (Collins *et al.* 1985). Such treatment will reduce the numbers of mycobacteria present in the sample by at least 5 to 10-fold, while killing other bacteria and yeasts to a greater extent. However, the compromise is more difficult to achieve in practice for stool specimens than for respiratory tract specimens which are usually less heavily contaminated.

In most cases, the intervals between receipt of different isolates from an individual patient were generally quite short. This may be expected since disseminated MAC infection usually occurs towards the end of the course of HIV infection when CD4 lymphocyte counts are low and life expectancy is short (section 1.3.1). Surprisingly, however, multiple isolates of MAC were received over a long period of time, up to 30 months, in a significant proportion of cases, and typing showed that the same strain (per patient) was responsible for persistent infection throughout. As discussed below however, the possibility of polyclonal infection in these patients, with the strain isolated representing the one best adapted to growth *in vitro*, was not excluded. The strains from patients with chronic infections had different PFGE types suggesting that a common strain of reduced virulence was not responsible. However, relatively little

is known about virulence factors in MAC (section 1.3.4), and such factors would not necessarily correlate with DNA restriction sites. Host factors that might predispose to persistent MAC infection are worthy of further study. These include HIV viral load and CD4 lymphocyte count at the time of infection, the level of tissue involvement and bacteraemia (colony forming units per gram or ml), although the latter does not correlate closely with symptoms in individual patients, and the presence of other HIV-associated disease.

Although stored under the same conditions, the viability of the MAC isolates on subculture was much better than that of the *M. tuberculosis* isolates. No correlation was found between the chance of successful subculture and the age of the stored slope. This means that libraries of MAC isolates may easily be stored for future studies. The apparently superior survival of MAC under conditions of nutrient limitation *in vitro* is probably related to the cell wall structure of this species. The mycobacterial cell wall and envelope contains several insoluble macromolecular components: arabinogalactan, peptidoglycan and mycolic acid, as well as lipoarabinomannan and, specific to MAC, the glycopeptidolipids which confer serological specificities. The complex array of hydrocarbon chains of some of these components is likely to be responsible for the impenetrability of the cell wall (Inderlied *et al.* 1993). This, in turn, may explain the ability of organisms of the MAC to survive in the environment. It may also account for intrinsic resistance to many antimicrobial drugs which are unable to penetrate into the cell (Rastogi & Barrow 1994), and for the difficulty in achieving cell lysis when attempting to extract intact mycobacterial DNA. The relative heat resistance of MAC, exemplified by

pseudo-outbreaks that have occurred in association with inadequate heating of sampling needles in BACTEC systems (Vannier *et al.* 1988, Bignardi *et al.* 1994, Burki *et al.* 1995), may also be due in part to the cell wall structure.

As other studies have shown, PFGE of the large restriction fragments generated by restriction digestion of chromosomal DNA with *Ase* I gave rise to a high degree of polymorphism among clinical isolates of MAC (Arbeit *et al.* 1993, Mazurek *et al.* 1993). This technique is highly discriminating for use as a typing method, in contrast to RFLP analysis using the probe pMB22 which appears to recognise only a limited number of strains (Hampson *et al.* 1989, Visuvanathan *et al.* 1992). The considerable genetic diversity of MAC isolates from AIDS patients is compatible with the hypothesis that infection is acquired from environmental sources. Since the laboratory work for this study was completed, RFLP analysis using the insertion sequence *IS1245* has been shown to generate a suitably high number of copies and a diversity of patterns comparable to that obtained with PFGE for human strains of *M. avium* (Guerrero *et al.* 1995). In view of the difficulty in reliably achieving lysis of the cell wall to obtain intact genomic DNA in this species, and the lack of a stable end product with PFGE (that is, an agarose gel rather than DNA cross-linked to a nylon membrane), *IS1245* RFLP analysis may prove to be a more popular method for future studies involving typing of *M. avium* strains. However, its value will depend on the number of copies and heterogeneity of position of *IS1245* in the particular collection of strains under investigation. For example, von Reyn *et al.* (1996) have encountered epidemiologically unrelated strains with distinct PFGE profiles but indistinguishable *IS1245* RFLP patterns containing three or fewer copies of the insertion element. As

with RFLP methods using other probes, if a high proportion of strains contains few copies then other methods will be required to subdivide isolates which appear to cluster together.

Guidelines for interpreting chromosomal DNA restriction patterns produced by PFGE in the context of bacterial strain typing have been published (Tenover *et al.* 1995). These are most relevant to comparatively small sets of isolates related to putative outbreaks of infection. Isolates are designated as indistinguishable if their restriction patterns have the same number of bands of the same apparent size. They are closely related if two or three fragments differ compared to the outbreak pattern; such changes result from a single genetic difference compared to the outbreak strain, for example a point mutation leading to gain or loss of a restriction site, or the insertion or deletion of DNA in an existing fragment. Strains with two genetic differences have four to six fragment differences compared to the outbreak strain and are regarded as only possibly related to the latter. In this study, however, almost all isolates appeared to be either unrelated or indistinguishable to each other.

PFGE has been criticised previously for poor reproducibility from one experiment to another (Saunders & Ridley 1994), but this was not found in this study. Under the same electrophoretic conditions (voltage, duration, switch times) and composition of buffer and gel, a strain of MAC gave reproducible patterns from gel to gel.

However, only one apparatus was used here by one operator, so the reproducibility of the method in different laboratories remains to be established. There is a need for a standardised methodology so that results from different laboratories may be compared,

as has been established for IS6110 RFLP analysis of *M. tuberculosis*. If libraries of stored MAC strains are established, application of the standardised methodology may then lead to a greater understanding of the molecular epidemiology of this infection.

Typing multiple isolates of MAC from the same patient could suggest clues about the pathogenesis of this infection. For example, isolates from different body sites that appear to be the same strain by typing are likely to be the result of disseminated infection. Isolation of a strain from a superficial site such as the gastrointestinal or respiratory tract before it is subsequently isolated from blood cultures, is consistent with colonisation of the site preceding invasion and disseminated infection. However, a prospective study in which frequent cultures from different body sites are made, in susceptible patients without a previous history of MAC, is really required to be able to draw valid conclusions about pathogenesis. In the present study, isolates were not necessarily referred by source laboratories in the same order that cultures were taken from the patients. This may be because, for example, a blood culture inoculated into liquid medium usually yields growth before a sputum sample taken at the same time, but cultured on solid medium after a decontamination step likely to reduce the number of viable organisms present. From a minority of patients, two or more genotypically different strains of MAC were isolated. In this group stool isolates rather than those from respiratory tract samples more frequently differed from the blood/bone marrow isolates. This observation supports the view that colonising strains and those responsible for disseminated infection in the same patient may be different, which is not surprising if there is continual exposure to multiple strains in the environment. Havlik *et al.* (1994) have demonstrated that screening cultures for patients at risk of

disseminated MAC infection have little predictive value because in some cases colonisation may not be detected before dissemination occurs, and in others established colonisation does not progress to dissemination. Although numbers are small, one interpretation of the above results is that strains colonising the respiratory tract are more likely to lead to disseminated infection than those present in the gut. An alternative hypothesis is derived from autopsy findings in 44 AIDS patients with MAC bacteraemia (Torriani *et al.* 1994). 30% of these patients had no histological evidence of MAC and the risk of developing detectable tissue involvement was proportional to the duration of bacteraemia. The authors favoured the view that invasion of a mucosal surface by MAC is followed by transient bacteraemia during which the organism is seeded in multiple organs eventually giving rise to extensive tissue disease and increasing levels of bacteraemia. If this is the case, organisms present in say the lungs may represent the eventual target rather than the original source of the infection.

Several studies in the United States have concluded that polyclonal infection with MAC, that is two or more different strains simultaneously causing disseminated infection, is common among patients with AIDS (Arbeit *et al.* 1993, Slutsky *et al.* 1994). Although unequivocal evidence for polyclonal infection was not obtained in this work, it is possible that use of stored slopes of referred isolates rather than primary cultures may have underestimated the occurrence of this phenomenon. In order to detect polyclonal infection clinical specimens need to be plated on primary isolation media in such a way that the individual colonies subsequently grown can be picked off for subculture and typing independently. Mixed cultures are not readily

apparent when growth is confluent on Lowenstein-Jensen slopes. (The same criticism could also be made of cultures of *M. tuberculosis* referred to the laboratory.

However, there is little evidence to suggest that polyclonal infections are common in tuberculosis.) Furthermore, most of the source laboratories in this study employed liquid media (such as BACTEC) for culturing blood rather than solid media (as in the Dupont Isolator lysis centrifugation system). It is possible, as von Reyn *et al.* (1996) have discussed, that when two strains of MAC are inoculated together into a liquid medium, one may compete more effectively and grow more rapidly at the expense of the other, giving the impression of a single strain by the time that the threshold of a positive growth index is reached in the bottle. Primary isolation on solid media is also important for drug sensitivity testing, because in a polyclonal infection overgrowth by a susceptible strain in liquid culture may potentially mask the presence of a second drug resistant strain. In a small number of patients, isolates from positive blood cultures referred several months apart were genotypically different. These were thought to be consecutive rather than true polyclonal infections, as the different strains were not present simultaneously. However, it is possible that sampling error resulted in the recovery of a different strain on each occasion from a polyclonal bacteraemia.

Two clusters of cases infected with indistinguishable MAC strains were discovered. Epidemiological information about the patients involved is incomplete, but in the larger cluster, patients had attended several hospitals and two different convalescence homes and no single common factor could be identified. Furthermore, many other AIDS patients attended these units over the same time period but were infected with different strains of MAC. Most of the patients in the cluster had received

chemotherapy for cytomegalovirus retinitis or malignancy. This would have resulted in further immunosuppression, perhaps increasing the susceptibility of the patients to disseminated MAC infection. However, other patients undergoing these treatments were not infected by the same strain of organism.

A study of MAC strains isolated from 129 patients in Berne, Switzerland, although uncovering significant problems due to cross-contamination in a BACTEC system, reached similar conclusions with regard to the diversity of genuine MAC isolates in the community (Burki *et al.* 1995). Most patients had unique strains as judged by PFGE pattern. However, several clusters of two and three cases were noted, as well as a group of eight HIV patients from whom a strain with the same PFGE pattern was isolated, mostly from respiratory tract specimens. Further epidemiological information on this group was not given however. The particular strain was estimated to account for one fifth of MAC isolated from HIV patients in that area (Burki *et al.* 1995).

The long interval of approximately 18 months between the first and last identified cases in the larger cluster of eight cases could be explained by continuing exposure of susceptible patients to a common source of the 'outbreak' strain. Recirculating hot water systems were suggested to be such a source in two small groups of patients (3 and 2 cases respectively) at two hospitals in Boston and New Hampshire (von Reyn *et al.* 1994). The hot water systems at these institutions were found to be persistently colonised with the same strain of MAC responsible for disseminated infection in the patients concerned, who had had numerous possible exposures to hospital hot water.

Ideally, water samples from the hospitals and convalescence units attended by cases in the present study would be cultured for mycobacteria, to see if the same strain of MAC could be recovered. However, water samples are not available from the hospital which treated the majority of these patients (Westminster Hospital) which was closed in 1993 when the unit moved to new premises. This may be a suitable area for further study, especially as it has been suggested that disinfection of hospital hot water distribution systems may prevent some MAC infections in AIDS patients (Singh & Yu 1994, von Reyn *et al.* 1994). As exposure to multiple sources of MAC in the environment is the rule for most persons, such a measure cannot be expected to prevent all infections. However, the alternative of prophylaxis with antimycobacterial drugs such as clarithromycin (Pierce *et al.* 1996), azithromycin and/or rifabutin (Havlir *et al.* 1996), while improving survival may also lead to adverse effects, problems with drug interactions, and development of drug resistance (Horsburgh 1996).

5. CONCLUSIONS

The pandemic of HIV infection has had considerable impact on disease caused by mycobacteria. In some parts of the world notifications of tuberculosis have risen largely as a result of immunosuppression caused by HIV. Differences in the presentation of tuberculosis, including the prominence of extrapulmonary disease and accelerated progression of infection are also apparent. *M. avium* complex, a previously somewhat neglected organism, has also come to prominence as disseminated infection by this organism is a common opportunistic infection in AIDS patients and is seen almost exclusively in them. While the mode of transmission and pathogenesis of tuberculosis are well understood (although the extent of reactivation versus recent transmission among HIV patients in the United Kingdom has received less attention), such fundamental aspects of MAC infection remain to be fully established.

Building a library of stored isolates from patients and analysing them with an appropriate genotyping method is one approach to improve understanding of the molecular epidemiology of these infections. In this study, isolates from HIV patients referred from laboratories in South East England to Dulwich Public Health Laboratory between 1991 and 1994 were used. The viability of *M. tuberculosis* strains stored on Lowenstein-Jensen slopes at room temperature was found to be low after two years. Strains are now stored at -20°C as a result. By contrast, MAC isolates survived well at room temperature.

RFLP analysis with a probe to insertion sequence IS6110 was used for *M. tuberculosis* and was easily set up in a clinical laboratory which handles primary

isolates and referred strains of this organism. PFGE of *Ase* I large restriction fragments was used as the method for typing MAC. Both methods were reproducible and had adequate discriminatory power for the strains studied. IS6110 RFLP analysis has the advantage that a standardised methodology has been agreed, enabling comparison of results between different laboratories and the potential for establishing a computer database of results. If PFGE is to become established as the main method for typing MAC, standardisation of the methodology will be required.

Isolates of *M. tuberculosis* from 96 HIV seropositive patients and 84 controls were examined. As expected, those from HIV patients were more often from extrapulmonary sites compared to the control group. Resistance to one or more first line antituberculous drugs also occurred more frequently among the isolates from HIV patients. However, only two strains resistant to both rifampicin and isoniazid (that is, MDRTB) were encountered in this group, from unconnected patients. IS6110 typing revealed considerable heterogeneity among the isolates from HIV patients, and the most likely explanation for this is the diversity of social and ethnic origins of such patients living in South East England, many of whom acquired their original *M. tuberculosis* infection elsewhere. Nevertheless, four clusters of cases, involving a total of twelve patients, with strains having indistinguishable RFLP patterns were observed. This was significantly more than among the control group and suggests that recently transmitted infection is responsible for clinical disease more frequently in HIV patients. This conclusion is consistent with the findings of studies in other parts of the world. In the largest cluster of five cases epidemiological information supported the conclusion that nosocomial transmission of tuberculosis had occurred

from one HIV patient to two others. A healthcare worker at the same hospital, who was not known to be HIV positive, later presented with clinical disease due to the same strain. This was the first outbreak of tuberculosis in an HIV care centre described in the United Kingdom. These clusters of cases all involved drug sensitive strains, but two outbreaks of MDRTB in London have subsequently been reported.

56 AIDS patients with disseminated MAC infection were studied. Ase I large restriction fragments of the strains isolated from different patients showed considerable heterogeneity. This is consistent with the findings of other studies that have used PFGE to type isolates of MAC. Most of the patients who had isolates from more than one body site, as well as those in whom infection persisted for twelve months or more, had the same strain throughout. No cases of concurrent polyclonal infection were seen. However, the use of cultures from stored slopes in this study together with the widespread employment of liquid culture media for detecting disseminated MAC infection in referring laboratories means that the proportion of cases with polyclonal infection may be underestimated. A prospective study involving frequent surveillance cultures from patients at risk of disseminated MAC infection, with typing of individual colonies isolated on solid media, is required to further elucidate the pathogenesis of this condition. A cluster of eight patients with indistinguishable MAC strains was observed, although insufficient epidemiological information was available to confirm that this was due to an outbreak of infection.

An important aim of molecular epidemiological work is to apply the results in order to prevent future infection in susceptible individuals. For *M. tuberculosis*, it is clear that

attention to infection control precautions, in particular isolation of patients suspected of having pulmonary tuberculosis and the use of rooms with negative pressure ventilation for performing aerosol-generating procedures, is the key to controlling transmission of infection. If further evidence is obtained of common sources of MAC, such as hospital hot water supplies, appropriate treatment of these sources may prevent a proportion of MAC infections in AIDS patients. As the organism is widely present in the environment however, chemoprophylaxis may be a more effective alternative at present.

Many unanswered questions remain, particularly regarding the pathogenesis of disseminated MAC infection, which is a deserving area for future research.

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